REMARKS

The Office Action of October 30, 2003 presents the examination of claims 83-121. These claims remain pending. Claims 93 and 94 are objected to, but indicated as allowable if rewritten into independent form incorporating the limitations of the base claims.

Telephone interview

On July 7, 2004, a telephone interview was held with the Examiner to attempt to resolve the issues outstanding in this application. In that interview, the Examiner suggested some claim amendments that would place the application into condition for allowance, and she provided a copy of a reference from which text was to be taken to remedy a problem of improper incorporation by reference.

The present paper reflects the substance of that telephone interview. However, Applicants have not made all of the amendments recommended by the Examiner.

In particular, the Examiner had requested that the limitations of claim 84 be put into claim 83, and that "lentivirus" vectors be removed from the subject matter of the claims as not enabled. Applicants decline to make this amendment, presenting their detailed reasons below. However, new claim 124 effects the Examiner's suggestion in its entirety.

With respect to claim 83, Applicants have placed the approximate subject matter of claim 84 into the claim; a shorter piece of DNA is recited. New claims 122 and 123 recite progressively longer pieces of DNA; new claim 123 effects the Examiner's suggestion.

Claim 84 is now amended to recite the promoter fragment in terms of correspondence to a portion of SEQ ID NO: 1.

The Examiner also inquired during the interview about the number of species of MLC-2 promoter that were known at that the time the present application was filed. Applicants thus note that the sequence of the chicken MLC-2 gene promoter and of the rat MLC-2 gene promoter were both known at the time the first priority application was filed. Furthermore, some sequence comparison between the two had been done and a few common elements between them had been identified. (See, Exhibit 3, M-D. Zhou et al., *Mol. Cell. Biol.* 13:1222-1231 (1993), attached, in the introduction.)

Finally, the Examiner asked that Applicants' Representative review the Oath/Declaration of the Inventors to confirm that no priority claim was made to any German application filed more than one year previously to the International Application PCT DE96/02181, filed Nov. 14, 1996. Applicants' Representative notes the Supplemental Declaration filed on February 22, 1999, which claims priority under 35 USC § 119 of DE 195 42 838.2 filed on Nov. 17, 1995 and DE 196 60 630.7, filed on October 1, 1996. Thus, no

priority claim is made to any application filed more than one year prior to the International Application.

Replacement sheets for Figure 10

The Examiner indicates that Figure 10 is missing from the file copy of the specification. Replacement sheets for Figure 10 are provided herewith.

No new matter is added by the Replacement Sheets. The Sequence data of Figure 10 are present in the originally filed Sequence Listing. Features of the Sequence set out by underlining are described at the paragraph bridging pp. 16 and 17 of the originally filed application.

Amendments to the specification

The specification is amended to incorporate the text of the article by Stratford-Perricaudet as required by the Examiner.

Amendments to the claims

Claims 83, 84, 89, 91, 93, 94 and 96 are amended.

Claims 93 and 94 are amended to independent form as recommended by the Examiner.

Claim 83 is amended to describe the embodiment that the portion of the mammalian MLC-2 promoter that is utilized is that from -19 to -800 from the start of transcription, and new claims

122 and 123 are added to recite progressively larger fragments of the promoter. Claim 123 recites the original scope of claim 84 that was suggested by the Examiner. These amendments to the claims are supported by the text in the last paragraph on page 4 of the English translation of the specification.

Claim 84 is amended to recite that the promoter fragment is one corresponding to nucleotides 158 to 2784 of SEQ ID NO. 1. This amendment is supported by the specification in the Figure 1, showing the insertion of the MLC-2 gene promoter fragment beginning at a Bam HI site, which site is shown in Figure 10 at nucleotide number 158, and by Figure 10, showing the transcription start site, *i.e.* the end of the promoter fragment, as nucleotide 2406.

Claim 91 is amended to insert the word "terminal" as recommended by the Examiner to clarify the meaning of the term "repeat".

Claims 89 and 96 are amended to correct a minor typographical error ("aprotein").

Rejection under 35 U.S.C. § 112, second paragraph

Claims 91, 92, 95-97, 101-103, 107-109, 113-115, 119 and 120 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. The Examiner states that the term "repeat sequences" in claim 91 is unclear, as many sequences can be repeated. The Examiner recommended insertion of the word

"terminal" as remedial to identify the specific repeat sequence that was intended. This amendment has been made to correct the oversight, thus obviating this rejection.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 83-92 and 95-121 remain rejected under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement. This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

The Examiner's position is that the present invention is well-enabled as to adenovirus and adeno-associated virus vectors, but that the state of the art of lentivirus vectors was not sufficiently developed to include this family of vectors within the claims.

As a threshold matter, Applicants note that new claim 124 does not recite lentivirus vectors and is otherwise commensurate in scope with subject matter the Examiner has deemed allowable.

The Examiner undertakes analysis of the question of whether undue experimentation is required to practice the present invention using a lentivirus vector by referring to several publications that purport to show that many problems exist in successfully transforming cells with a lentivirus vector to achieve sufficient expression of a desired gene. Palu is cited as describing the problem of obtaining sufficient tissue targeting and expression level. Applicants note that the present specification addresses the tissue targeting issue by teaching that direct injection of the vector into cardiac muscle or into the cardiac cavity leads to infection of the desired cells. Furthermore, the present

specification addresses the problem of achieving sufficient expression level by utlilizing a promoter demonstrated in a working example to drive cardiac muscle cell-specific expression of a desired gene at levels sufficient for detection of the protein, although the time course of the expression is not described. Applicants assert that the present specification directly addresses at least two of the three problems of gene therapy that Palu raises.

Peracchi is cited as describing the problem of cellular uptake of nucleic acids and the deficiencies seen in the art when ribonucleic acids are presented to cells. Peracchi describes application of "bare" nucleic acids to cells.

This reference is in large degree irrelevant to the present invention as directed to lentivirus embodiments. Lentiviruses can be administered as whole viral particles, and the tropism of the viral particle can be mediated by packaging the vector nucleic acid in the appropriate cell line. Applicants note the teachings of Naldini et al., *Science* 272:263 ff. (1996), cited by the Examiner, which describe pseudotyping of a lentivirus construct to produce particles expressing a VSV G envelope protein. These viral particles are shown to infect diverse cell types of fibroblasts and neuronal cells. Naldini et al. also describe vector components that provide for efficient packaging and sufficient viral titer.

To the degree that the Examiner suggests that direct injection of a lentiviral vector would not be effective in delivery of a construct according to the invention, Applicants provide attached Exhibit 4, Kafri, T. et al., *Nature Genetics* 17:314 (1997). Though published about one year after the priority date of the instant application, Kafri et al. evidences that, as asserted in the instant specification, direct injection of lentiviral vectors into muscle tissue transduces cells of the injected tissue at a sufficient rate to provide efficient transduction and expression of the desired gene.

The Examiner further argues that:

> Lentiviral biology was still in its infancy at the time the invention was made. ... The utilization of various lentiviral components for the construction of retroviral vectors required undue experimentation beyond that provided in the instant disclosure at the time the invention was made, for the various components of different lentiviruses were not found to be analogous or interchangeable (*e.g.* see the abstract of Rosin-Arbesfeld...)

This argument is inapposite. The Examiner is reminded that the technical matter of the present invention relates to the MLC-2 promoter and the portions of it that provide cardiac muscle-cell specific transcription. The promoter of the present invention can be combined with a desired structural gene and then the construct is placed into a vector for delivery. The present invention is not about developing entirely new vectors for delivery. Rather, the

present inventors realize that cardiac muscle tissue consists largely of non-dividing tissue and therefore the ability of lentiviral vectors to transduce non-dividing cells (*see*, Naldini, *supra*) is useful in the present invention. Applicants are not claiming any and all lentiviral vectors *per se*, only those containing the promoter described as the instant invention. Thus, description of the components of an effective lentiviral vector other than the construct to be expressed in the cardiac tissue is unnecessary in the present specification.

The Examiner next asserts that the specification provides no description of construction of any lentivirus vector comprising the promoter of the invention, nor any working example thereof. As explained above, the specification directly states that a lentivirus vector is useful for delivery of constructs of the invention. The claimed invention relates to vectors comprising the promoters that are well-described and enabled in the present specification. The particulars of how to insert that promoter, together with a structural gene to be expressed, are considered by the Applicants to be known from the state of the art at the time the invention was made. In this regard, Applicants can point to Naldini et al., *Science* 272:263 ff. (1996), cited by the Examiner, who have utilized a lentivirus vector to express a beta-galactosidase gene in brain tissue transduced by *in vivo* injection of a recombinant lentivirus vector. As further evidence that

functioning lentivirus vector systems were known in the art, Applicants provide attached hereto Exhibits 5-7, a collection of papers:

Buchschacher Jr. et al., *J. Virology* 66:2731 (1992),

Carroll, R. et al., *J. Virology* 66:6047 (1994),

Naldini, L. et al., *PNAS USA* 93:11382 (1996).

Each of these papers describes a lentivirus vector system and insertion of a desired construct for transduction into cells. Thus, to the degree such is deemed required for enablement, this aspect of the present invention was well-established in the art at the time the present invention was made.

Finally, the Examiner argues that the breadth of the claims and quantity of experimentation required to practice the invention are large. The Examiner's position is that "de novo" development of a lentivirus vector system is required to practice the invention throughout the scope of the claims as represented by the term "lentivirus", and that the art is very unpredictable. The essence of the Examiner's argument is that the various parts of the lentiviral genome that should be deleted for insertion of the constructs according to the invention and control of infectivity of the lentivirus vector are not described in the instant specification. Applicants submit that such is not required, as several operable lentivirus vectors were known in the art at the

time the instant application was filed, as demonstrated by the evidence of Exhibits 5-7.

Applicants submit that the full scope of the present claims is adequately enabled by the specification. No undue experimentation is required to practice the lentivirus embodiments of the present invention. Accordingly, the rejection of claims 83-92 and 95-121 under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement should be withdrawn.

The present application well-describes and claims patentable subject matter. The favorable action of allowance of the pending claims and passage of the application to issue is respectfully requested.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Mark J. Nuell (Reg. No. 36,623) at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____
Mark J. Nuell, #36,623

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

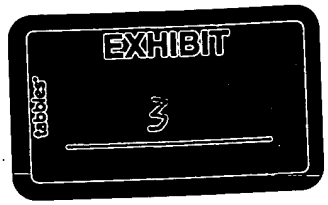
DRN/mua
0690-0110P

Attachment(s): Three (3) Replacement Sheets
Exhibits 3-7

AMENDMENTS TO THE DRAWINGS

The Examiner indicates that Figure 10 is missing from the file copy of the specification. Replacement sheets (FIGS. 10A-10C) for Figure 10 are provided herewith.

MOLECULAR AND CELLULAR BIOLOGY, Feb. 1993, p. 1222–1231
0270-7306/93/021222-10$02.00/0



Vol. 13, No. 2

A New Serum-Responsive, Cardiac Tissue-Specific Transcription Factor That Recognizes the MEF-2 Site in the Myosin Light Chain-2 Promoter

MING-DONG ZHOU, SHYAMAL K. GOSWAMI, MARY ELLEN MARTIN, AND M. A. Q. SIDDIQUI*

Department of Anatomy and Cell Biology, State University of New York Health Science Center, Brooklyn, New York 11203

Received 7 February 1992/Returned for modification 14 April 1992/Accepted 20 November 1992

We have identified a serum-responsive, cardiac tissue-specific transcription factor, BBF-1, that recognizes an AT-rich sequence (element B), identical to the myocyte enhancer factor (MEF-2) target site, in the cardiac myosin light chain-2 (MLC-2) promoter. Deletion of the element B sequence alone from the cardiac MLC-2 promoter causes, as does that of the MEF-2 site from other promoters and the enhancer of skeletal muscle genes, a marked reduction of transcription. BBF-1 is distinguishable from cardiac MEF-2 on the basis of immunoprecipitation with an antibody which recognizes MEF-2 but not BBF-1. Unlike MEF-2, BBF-1 is present exclusively in nuclear extracts from cardiac muscle cells cultured in a medium containing a high concentration of serum. Removal of serum from culture medium abolishes BBF-1 activity selectively with a concomitant loss of the positive regulatory effect of element B on MLC-2 gene transcription, indicating that there is a correlation between the BBF-1 binding activity and the tissue-specific role of the element B (MEF-2 site) sequence. The loss of element B-mediated activation of transcription is reversed following the refeeding of cells with serum-containing medium. These data demonstrate that cardiac muscle cells contain two distinct protein factors, MEF-2 and BBF-1, which bind to the same target site but that, unlike MEF-2, BBF-1 is serum inducible and cardiac tissue specific. BBF-1 thus appears to be a crucial member of the MEF-2 family of proteins which will serve as an important tool in understanding the regulatory mechanism(s) underlying cardiogenic differentiation.

The expression of cardiac muscle genes during myogenesis is influenced by a plethora of factors, including serum and growth factors (15, 18, 23, 27, 40, 45). Although recent work has established that these agents exert their effects directly, or indirectly, on transcription of target genes, the molecular events involved in production of the cardiac phenotype and differentiation of the cardiac myoblast remain to be elucidated. The expression of cardiac muscle genes is activated by serum and serum growth factors in culture medium (40, 45), while removal of growth factors is obligatory for differentiation and activation of gene transcription in skeletal muscle cells (14). Serum induces an early growth response gene, Egr-1, which can *trans*-activate the cardiac α-myosin heavy chain (α-MHC) gene in a transfection assay (18). In general, serum-induced regulation of muscle-specific genes is mediated by serum response elements and serum response element-related sequences which serve as target sites for binding of serum response factors (SRF) and SRF-related factors (36 and references therein), suggesting that serum and serum growth factors play a pivotal role in control of myogenic differentiation.

The tissue-specific expression of cardiac muscle genes is mediated by several *cis* elements in the respective genes and the cognate *trans*-acting regulatory factors (15, 18, 22, 41, 51, 52). The lack of expression of the chicken cardiac myosin light chain-2 (MLC-2) gene in skeletal muscle cells is due to a negative regulatory mechanism mediated by an upstream promoter element, CSS, and its binding proteins present in skeletal muscle cells (41). Another group of researchers (33, 52) has described an activator element (HF-1) in the rat cardiac tissue-specific MLC-2 promoter which is implicated in cardiac tissue-specific gene transcription. A 47-bp element in the cardiac troponin T (cTnT) gene containing a 10-bp AT-rich sequence, conserved in both chicken and rat cardiac MLC-2 genes, is also involved in cardiac tissue-specific transcription (22). However, proteins that recognize the AT element in the cTnT promoter are present in both muscle and nonmuscle cells.

We have recently described (38) an activator, element A (CCAAAAGTGG), in the cardiac MLC-2 gene similar to the evolutionarily conserved CArG sequence responsible for up-regulation of muscle genes (29, 30). The CArG box sequence, however, interacts with multiple nuclear proteins (19, 26, 29–31), including the ubiquitous SRF which are involved in activation of both skeletal and cardiac muscle gene transcription (2, 5, 19, 26, 31; also see reference 39 for a review). A second AT-rich sequence, element B, in the MLC-2 promoter is indistinguishable from the consensus (C/T)T(A/T)(A/T)AAATA(A/G) sequence present in a number of muscle gene promoters and enhancers which are recognized by the myocyte-specific enhancer binding factor (MEF-2), originally identified in extracts of a skeletal muscle cell line, C2 (17). The MEF-2 DNA site sequence is present, in addition to the muscle creatine kinase (MCK) enhancer (17), in rat cardiac MLC-2 (52), brain and muscle creatine kinases (21) and in human phosphoglycerate mutase gene promoters (32). Deletion of this element from the respective genes causes a drastic reduction in transcription (17, 22, 32, 51). A protein, designated TARP, binds to the AT-rich sequence in the brain creatine kinase gene and is presumably functionally interchangeable with MEF-2 (21). TARP is,

* Corresponding author.

however, found in both muscle and nonmuscle cell types (7, 21, 22).

Thus, there is no clear evidence for a single element or factor that would account for the existence of a common regulatory pathway for activation of cardiac muscle-specific gene transcription. Neither is there evidence for a cardiac tissue-specific SRF despite the fact that cardiac muscle genes respond to serum-mediated stimulation. The best-characterized muscle-specific regulatory factors to date are members of helix-loop-helix (HLH) proteins of the MyoD family, present in skeletal muscle cells which recognize a consensus E-box sequence found in muscle genes (3, 4, 12, 25; see references 34 and 39 for a review). Myogenin, a member of the MyoD family, is involved in regulation of MEF-2-dependent activation of the skeletal muscle MCK gene (9), suggesting a pivotal role for MEF-2 in the myogenic program. However, many muscle genes, like the cardiac MLC-2 gene, do not contain a functional E-box. Also, MyoD family proteins are not present in cardiac muscle cells as such; other regulatory pathways, independent of the MyoD–E-box requirement, must be operational in transcriptional activation of these genes. In this context, MEF-2 family proteins are potentially important in mechanisms underlying differentiation of these two muscle lineages. Specific MEF-2 isoforms, generated through alternatively spliced RNA, have recently been identified in skeletal and heart muscle cells and are implicated in tissue-specific function of the respective genes (49). In this paper, we describe a transcription factor, BBF-1, present in cardiac muscle cells, which binds with sequence specificity to the AT-rich MEF-2 binding site (element B) in the cardiac MLC-2 gene. BBF-1 activity is present in cardiac muscle nuclear extracts but is absent, or present at a barely detectable level, in skeletal muscle. BBF-1 is distinguishable from MEF-2 on the basis of immunoprecipitation with an antibody, SRF RSRFC4 (36), directed against the AT-rich site-binding protein, which recognizes MEF-2 but not BBF-1. BBF-1 activity disappears in cardiac muscle cells grown in serum-reduced culture medium with a concomitant loss of the positive role of element B in transcription. These studies thus suggest a mechanism whereby the tissue-specific activation of the cardiac MLC-2 gene appears to be the function of a member of the MEF-2 family of proteins, BBF-1, which responds to a serum-mediated signalling pathway in the regulation of cardiac MLC-2 gene expression.

MATERIALS AND METHODS

Cell culture. Heart and skeletal (thigh) muscle tissues excised from 13-day-old chicken embryos were used to prepare primary cell cultures as described previously (41). Dissociated cells, freed of fibroblasts by repeated differential plating, were cultured in growth medium F-10 (10% fetal bovine serum, 10% horse serum, 100 U of penicillin per ml, 100 U of streptomycin per ml). Skeletal muscle cells were grown in medium containing 10% horse serum–2% chicken serum–100 U of penicillin per ml–100 U of streptomycin per ml. Both cell cultures were plated at a density of 1.5×10^6 cells per 100-mm plate for transient transfection. To test the effect of serum concentration on cardiac muscle cells, the cells were cultured with high serum levels (10% FBS and 10% horse serum) for 2 days or as indicated otherwise and then shifted to low serum levels (3% horse serum).

Transfection of cells and CAT assay. Cells were transfected by the calcium phosphate precipitation method (16) as described earlier (41). Twenty micrograms of plasmid DNA per plate was used in each case, unless stated otherwise. Cells were harvested 56 h after transfection to prepare cell extracts for chloramphenicol acetyltransferase (CAT) assay (16). For serum induction, the cells were refed with high- or low-serum-concentration medium 16 h after transfection and then cultured for another 56 h. For normalization of DNA uptake, cotransfection of a plasmid, CMV-gal, containing the β-galactosidase reporter gene (1) was done and data (means ± standard deviations) were presented as percent activity of PSV2CAT, used as a positive control. Multiple plasmid DNA preparations were used for each experiment to ensure reproducibility.

Mutagenesis of the MLC-2 proximal promoter. 5′-deletion and site-directed mutants of the MLC-2 promoter were constructed as described previously (38, 50, 51). The parent plasmid, pLC106CAT (50), which contains a 1.3-kb MLC-2 gene promoter and the 5′-flanking region, was linearized by *Xho*I, digested by *Bal* 31, and terminated at different time points to obtain different lengths of the promoter. The DNA was digested with *Sma*I, and the large fragment of the digestion product was ligated with *Bal* 31 products and transformed into *Escherichia coli* JM109. Site-directed mutants were obtained from polymerase chain reaction products synthesized with oligonucleotide primers containing a *Kpn*I site (CTCGAG) displacing the target sequence and the two 5′ and 3′ distal primers including *Nde*I or *Hin*dIII sites as described previously (51). The recombinants were identified by restriction endonuclease digestion and DNA sequencing analysis.

Preparation of nuclear extracts. The nuclear extracts were prepared essentially according to Dignam et al. (11) and as described previously (41). The protease inhibitors leupeptin (1 μg/ml) and pepstatin (1 μg/ml) were used routinely. The extracts usually contained 4 to 6 mg of protein per ml and remained stable in liquid nitrogen for several months. To compare the serum effect on cardiac nuclear proteins, the extracts were prepared from myocytes cultured for 5 days, 2 days with high serum levels and 3 days with low serum levels for low-serum extracts and 5 days continuously with high serum levels for high-serum extracts.

Gel shift assay. Double-stranded DNA fragments obtained by renaturation of chemically synthesized oligonucleotides were radio-labeled at the 5′ end by polynucleotide kinase and [^{32}P]ATP and used for the binding assay. DNA fragments (5,000 cpm) and nuclear proteins (10 μg) were incubated in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) (pH 7.9)–50 mM KCl–5 mM $MgCl_2$–0.5 mM EDTA–1 mM dithiothreitol–12.5% glycerol at 4°C for 90 min and were separated on the 8% polyacrylamide gel at 4°C as described earlier (41, 51). For competition, the unlabeled DNA in 100-fold excess was added to the reaction mixture before the addition of nuclear extracts. For immunoprecipitation assay, 0.5 μl of preimmune serum or antiserum of RSRFC4 was preincubated with 10 μg of nuclear extracts for 30 min at 4°C before incubation with the DNA probes as analyzed, as described above.

RESULTS

The AT-rich element B is a cardiac-tissue specific activator of MLC-2 gene transcription. In previous studies (38, 51), we have demonstrated that the AT-rich elements A and B (Fig. 1) are muscle-specific activators as these two elements caused stimulation of transcription following transfection of primary cardiac muscle cells but not of fibroblasts and brain cells. Element A is similar to the CArG box sequence (19,

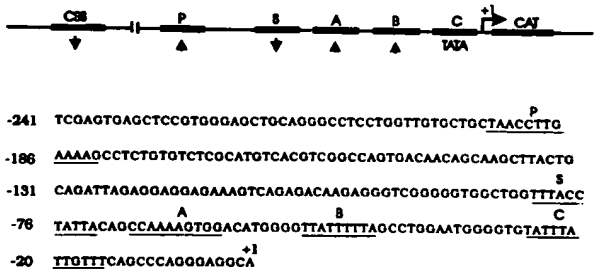


FIG. 1. Partial nucleotide sequence of the chicken cardiac MLC-2 gene promoter. Multiple *cis*-acting DNA elements, A, B, C, P, S, and CSS, are indicated (see text). +1 denotes the transcription start site. Arrowheads pointing upward denote positive roles and arrowheads pointing downward denote negative roles in transcriptional regulation.

30), and element B is indistinguishable from the MEF-2 site sequence in the MCK enhancer (17), both implicated in tissue-specific activation of transcription. To examine whether the regulatory role of element B is restricted to cardiac muscle tissue, 5′-deletion and site-specific mutants of the MLC-2 promoter fused to the CAT coding sequence were transfected simultaneously into primary cardiac and skeletal muscle cells in culture. The results shown in Fig. 2 confirmed that plasmid pLCΔ31CAT, containing element C (TATA box) alone, produced basal-level promoter activity, approximately equal in cardiac and in skeletal muscle cells, whereas pLCΔ53CAT, which includes element B (MEF-2 site), showed a fivefold increase in activity in cardiac cells but not in skeletal muscle cells. pLCΔ72CAT, which includes element A containing the CArG-like sequence (CCAAAAGTGG), caused a further 3.5-fold increase in CAT expression, but in both cardiac and skeletal muscle

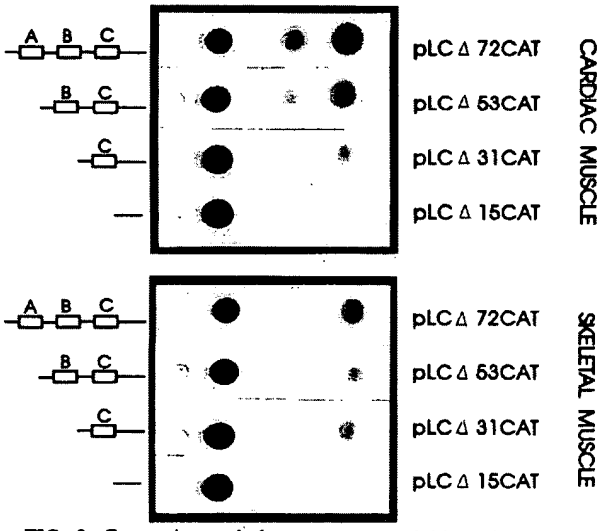
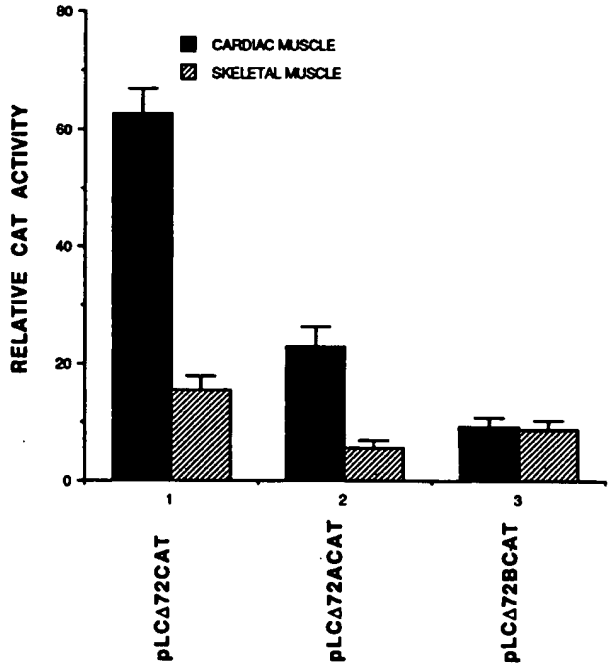


FIG. 2. Comparison of the promoter activities of 5′-deletion mutants of the MLC-2 promoter in primary cardiac and skeletal muscle cells. The sequentially 5′-deleted MLC-2 promoters fused to the CAT coding sequence were used to measure promoter activity in primary cardiac and skeletal muscle cells in culture as described in Materials and Methods. A, element A, containing the CArG-like sequence; B, element B (MEF-2 site); C, element C (TATA box).

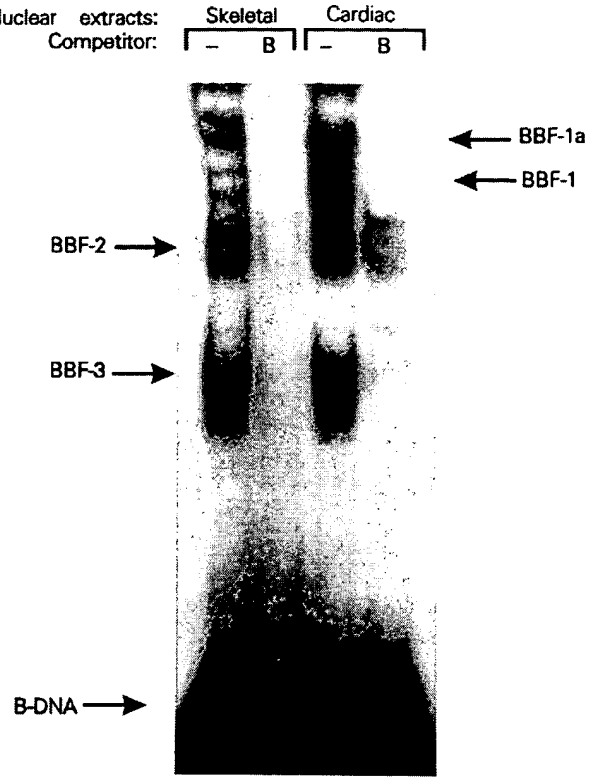


A B

pLCΔ72CAT: TACAGCCAAAAGTGGACATGGGGTTATTTTTAGCCT
pLCΔ72ACAT: TACAGCCCTCGAGGGACATGGGGTTATTTTTAGCCT
pLCΔ72BCAT: TACAGCCAAAAGTGGACATGGGGTGGATCCTAGCCT

FIG. 3. Comparison of MLC-2 promoter activity of site-specific mutants in cardiac and skeletal muscle cells. Deletion/substitution mutants of elements A and B were constructed by using the parent plasmid, pLCΔ72CAT, as described in Materials and Methods and transfected into primary cardiac and skeletal cells. CAT activity is expressed as a percentage of activity of pSV2CAT, used as a positive control, following normalization for DNA uptake as described in Materials and Methods. Each bar represents a mean value ± the standard deviation for 3 to 5 independent experiments. A and B, elements A and B, respectively.

cells, suggesting that element B-mediated activation of transcription is cardiac tissue specific whereas element A activates transcription in both types of muscle cells, consistent with the expected role of the CArG box sequence (29–31). We also made site-specific mutations in A and B sequences (see Materials and Methods) and tested for activity in cardiac and skeletal muscle cells. Data summarized in Fig. 3 indicated that the mutation of the element A sequence (pLCΔ72ACAT) caused a reduction in transcription levels in both kinds of cells as expected and yet the differential expression in cardiac and skeletal muscle cells was maintained. However, when the element B sequence was mutated (pLCΔ72BCAT), the reduction in transcription obliterated the differential expression in the two cell populations. Several precautions were taken (see Materials and Methods and references 38 and 41) to ensure reproducibility. The results were normalized for DNA uptake following cotransfection with plasmid CMV-gal containing the β-galactosidase coding sequence (1). Previous studies (33, 52) have identified a 28-bp sequence element (HF-1) in the rat cardiac MLC-2 gene which was implicated in cardiac tissue-specific activation of transcription on the basis of transcription in primary cardiac cells and in a soleus muscle cell line (Sol-8). Our studies have delineated at least two functional elements (A



B-DNA: GACATGGGGTTATTTTTAGCCTGGAATGGG

FIG. 4. Mobility shift assay with cardiac and skeletal muscle nuclear proteins. Nuclear extracts were prepared from cardiac and skeletal muscle tissues and analyzed for binding to a synthetic 30-bp oligonucleotide (−57 to −28) containing element B as described in Materials and Methods. The gel shift assay was performed without (−) or with a 100-fold molar excess of oligonucleotide B as competitor. The protein-DNA complexes are shown as BBF-1a, BBF-1, BBF-2, and BBF-3.

and B) within the HF-1-like segment in the chicken cardiac MLC-2 gene, one of which (element A), the CArG-like sequence, activates transcription in both cardiac and skeletal muscle cells, while the other MEF-2-binding site, element B, is cardiac tissue specific consistent with the activity of HF-1B in rat MLC-2 (33, 52). We have recently demonstrated that these two elements are recognized by different transcription factors and that the DNA-protein complexes produced in a gel shift assay are not blocked by each other (38, 51).

A cardiac tissue-specific transcription factor (BBF-1) recognizes element B. To examine whether the cardiac tissue-specific role of element B (MEF-2 site) is mediated through its interaction with a cardiac tissue-specific DNA-binding protein(s), nuclear extracts were prepared from both cardiac and skeletal muscle tissues and tested in a gel shift assay for sequence-specific binding to a 30-bp-long chemically synthesized oligonucleotide encompassing the sequence from −24 to −53 containing element B (MEF-2 site). At least four protein-DNA complexes, designated BBF-1a, BBF-1, BBF-2, and BBF-3, were formed with nuclear extracts from cardiac muscle (Fig. 4). This was consistent with the complex formation pattern with the DNA probe containing the MEF-2 site and the adjoining sequence with the rat cardiac myocyte extracts reported recently (32). One of the four complexes, BBF-1, was abundant in the cardiac extracts but was absent, or present only at a barely detectable level, in skeletal muscle tissue. When extracts were prepared from chicken skeletal primary cells in culture, BBF-1 was totally absent (see below). The extracts from cardiac and skeletal muscle tissues were prepared in parallel from the same embryos. The possibility that the lack of BBF-1 activity in skeletal muscle extracts was due to nonspecific inactivation of DNA-binding proteins was discredited by the fact that the intensities of BBF-2 and BBF-3 were unchanged.

Since the element B sequence is identical to the MEF-2 binding site of the MCK enhancer which produces a characteristic slow-moving complex with MEF-2 from the skeletal muscle cell line C2 (17), we asked whether BBF-1a, or BBF-1, is MEF-2. An antibody, RSRFC4 (a kind gift of R. Treisman), directed against the common carboxyl terminus of known serum-responsive DNA-binding proteins, which also recognizes MEF-2 (see below; 36, 49) was used in a gel shift assay. When preincubated with the cardiac nuclear extracts, the antibody eliminated the BBF-1a activity (designated MEF-2 from here on) (Fig. 5) without affecting BBF-1 or other element B-protein complexes. In a reciprocal experiment, nuclear extracts from skeletal muscle cell line C2C12 and the MEF-2 binding site DNA probe of the MCK enhancer were used (Fig. 6). The antibody inactivated MEF-2, which was also inhibited by element B DNA, demonstrating that MEF-2 and BBF-1a share the same DNA recognition site and are immunologically identical proteins. While only MEF-2 of the skeletal muscle C2C12 extracts was inhibited by element B DNA, both MEF-2 and BBF-1 of the cardiac extracts were inhibited effectively by MEF-2 DNA of the skeletal MCK enhancer (Fig. 7). DNAs containing the MyoD binding site (E-box) and the negative element S of the cardiac MLC-2 gene (51) (Fig. 1) were ineffective as competitors. Thus, the cardiac proteins contain at least two MEF-2-like factors with strict MEF-2 site sequence dependence, but one of the proteins, BBF-1, is different from MEF-2 on the basis of its lack of recognition by the antibody RSRFC4. We further examined the sequence requirements for BBF-1 and MEF-2 binding to element B by using DNA probes with mutations in the core element B (ΔB), 5′-flanking (5′ΔB), and 3′-flanking (3′ΔB) regions (Fig. 8). Clearly, both MEF-2 and BBF-1 require the core element B sequence as 5′ΔB and 3′ΔB, but not ΔB, inhibited the binding effectively. BBF-2, on the other hand, requires a sequence outside the mutated sequences, since it was inhibited by all three competitors. The binding properties of BBF-3 are presently unclear as it was only partially affected by the competitors.

Serum induces the expression of BBF-1 activity. It is well recognized that serum and serum growth factors regulate the expression of both cardiac and skeletal muscle genes (18, 40, 45). A conserved DNA binding site, $CC(A/T)_6GG$ (element A in cardiac MLC-2), the CArG box, is present in many muscle-specific and growth factor-regulated genes and binds to an SRF to activate both skeletal and cardiac muscle gene transcription (2, 5, 19, 26, 31, 44). It is believed that the SRF family consists of multiple proteins, which also recognize the AT-rich MEF-2 binding site (36, 49). MEF-2 appears in cultured skeletal muscle cells accompanying differentiation of the skeletal myoblast into myocytes due to serum deprivation (17). To ascertain whether the cardiac tissue-specific BBF-1 activity responds to modulation in serum concentra-

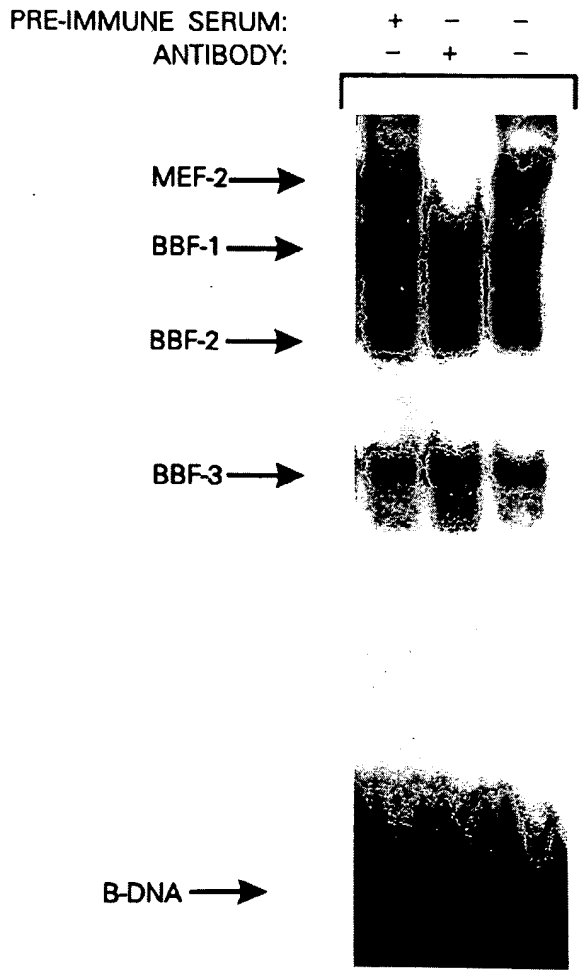


FIG. 5. Effect of an antibody, RSRFC4, on B-DNA and protein complex formation in a gel shift assay. A quantity of 10 μg of nuclear proteins from cardiac tissue was incubated with 0.5 μg of antiserum or preimmune serum of RSRFC4 for 30 min at 4°C or with neither, and the mixture was analyzed by gel shift assay with B-DNA as a probe as shown in Fig. 4.

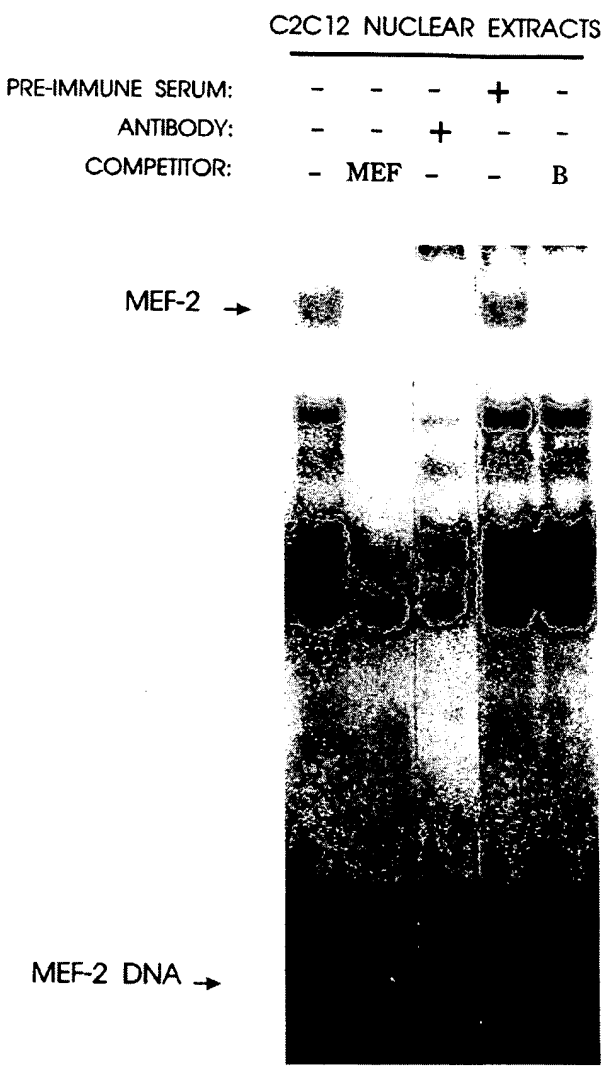
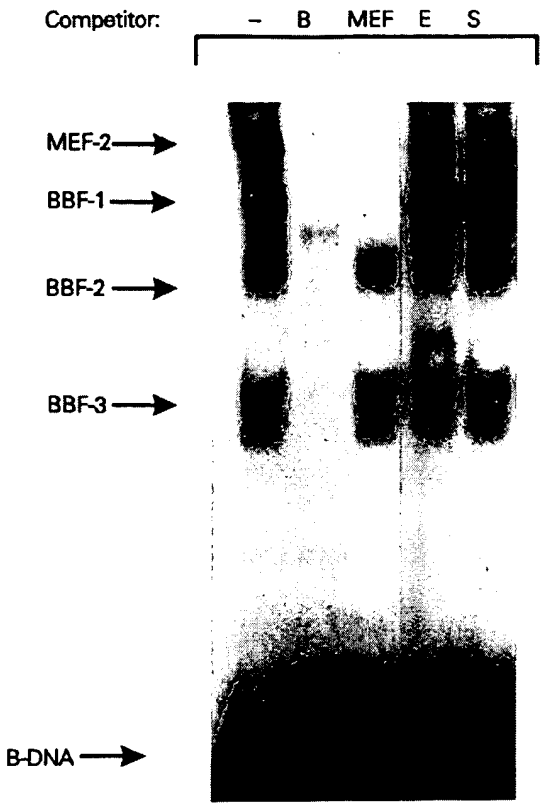


FIG. 6. Gel shift analysis of MEF-2 from C2C12 cells with RSRFC4 antibody. The antiserum of RSFRC4 was tested by preincubation with the nuclear extracts prepared from C2C12 myocytes in a gel shift assay with a probe containing the MCK MEF-2 site. The competitors used were MEF-2 and element B DNAs.

tion in culture medium, we tested BBF-1 binding to MLC-2 element B (MEF-2 site) and the MCK enhancer MEF-2 site as DNA probes in serum-stimulated and serum-deprived cardiac cells in culture. For this purpose, nuclear extracts were prepared from primary cardiac cells cultured in medium with high serum levels for 2 days, washed, and then switched to low-serum medium for 2 to 3 days or kept in high-serum medium continuously (see Materials and Methods). Three defined complexes were formed with element B and nuclear extracts prepared from the cardiac muscle cells cultured continuously in high-serum medium (Fig. 9). However, in extracts from the low-serum medium the slow-moving complex (BBF-1) (see arrow in Fig. 9) disappeared. MEF-2 site DNA produced only two complexes, one of which (see arrow) was also serum dependent (Fig. 9). Interestingly, the MEF-2 binding activity appeared to be at a minimum discernible level in both extracts cultured for 4 to 5 days. To confirm whether the continuously cultured cells are deprived of MEF-2, the extract from cells cultured for 5 days in high-serum medium (Fig. 10, I) was compared with extracts from cells cultured for 2 days in high-serum medium (Fig. 10, II) for binding to element B with and without preincubation with RSRF antibody, as before. Clearly, 2-day-cultured cells contain MEF-2 (BBF-1a) activity which was immunoprecipitable with the antibody. BBF-1 activity remained the same in both extracts. Although the loss of MEF-2 in prolonged cultures is of potential interest and would require further investigation, our data, presented in Fig. 9, nevertheless demonstrated unequivocally that BBF-1 activity is serum inducible.

BBF-1 target site (element B) is a serum response element. If BBF-1 is serum inducible, then its target sequence (element B or the MEF-2 site) must function in a serum-responsive manner. To demonstrate this, we transfected the mutant MLC-2–CAT recombinants into cardiac muscle cells after cells were grown in serum-containing medium for 24 h and then shifted to low-serum medium for the next 3 days. As

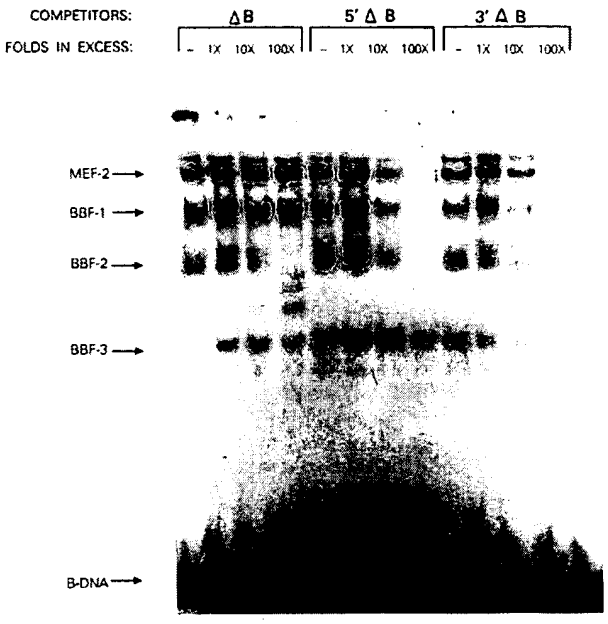


MEF-2: AGGGTTATTTTTAGAGCGAGCTTCTCCTCC

E: GATCCCCCCAACACCTGCTGCCTGAGATC

S: GCTGGTTTACCTATTACAGC

FIG. 7. Analysis of MEF-2 and BBF-1 by DNA competition. The specific binding of cardiac nuclear protein with element B was analyzed by a gel mobility shift assay using different oligonucleotides as competitors and nuclear extracts prepared from cardiac cells. The assay was performed in the absence of competitors (−) or with a 100-fold molar excess of B oligonucleotides, MEF-2 site (MEF), E-box (E), and S-element (S) DNA probes as competitors.



Δ B: GACATGGGGTGGTACCTAGCCTGGAATGGG

5'Δ B: GCTCGGGGTTATTTTTAGCCTGGAATGGG

3'Δ B: GTTATTTTTAGGGTACCATGGGGTGT

FIG. 8. Sequence requirements for MEF-2 and BBF-1 complex formation. Element B DNA and cardiac MEF-2 and BBF-1 complex formation was tested with DNAs containing a mutated core sequence of element B (ΔB) or a mutated 5'-flanking sequence (5'ΔB) and 3'-flanking (3'ΔB) DNAs as competitors used in 1, 10, and 100× molar excesses in a gel shift assay as described before. The sequences of mutated DNA probes are shown below.

shown in Fig. 11, the promoter activity of plasmid pLCΔ53CAT containing element B in cardiac muscle cells in low-serum medium was reduced significantly while the level of pLCΔ31CAT, the basal promoter, appeared to be insensitive to the serum shift. This was consistent with the disappearance of BBF-1 binding activity in extracts from cardiac muscle cells cultured in low-serum medium. These results also discredit the possibility that the loss of MEF-2 activity observed earlier in 5-day-old cardiac cell culture (Fig. 9 and 10) was due to fibroblasts or nonmyocytes which can overgrow myocytes. We have previously demonstrated that the cardiac MLC-2 promoter is totally inactive in fibroblasts and nonmuscle cells (51). The cultures used here promote MLC-2 expression optimally. More importantly, refeeding of the same cardiac muscle cells with high-serum medium after the shift to low-serum medium for 2 to 3 days allowed recovery of the promoter activity in pLCΔ53CAT, documenting that 5-day cultures contain predominantly myocytes. The serum-responsive regulation of MLC-2 promoter activity mediated by BBF-1, as is the case with SRF (36, 37, 44), is also reversible. Taken together, these results demonstrate that the AT-rich counterpart of the MEF-2 site in the cardiac MLC-2 gene promoter (element B) is recognized by at least two factors, MEF-2 and BBF-1. BBF-1 is different immunologically from MEF-2, and it displays tissue specificity and serum responsiveness.

DISCUSSION

In this report, we have presented evidence for the existence of a cardiac tissue-specific and serum-responsive nuclear factor, BBF-1, which mediates the activation of cardiac MLC-2 gene transcription through its interaction with an AT-rich MEF-2 site promoter sequence, element B. Deletion of this sequence, like that of the MEF-2 binding site from other promoters and enhancers of skeletal muscle genes (17, 21), caused a marked reduction of transcriptional activity, suggesting that element B (or the MEF-2 site) plays a pivotal role in both skeletal and cardiac muscle genes. Indeed, a recent report (32) suggests that a single MEF-2 binding site in the muscle-specific subunit of the human phosphoglycerate mutase gene accounts for the enhanced expression of the gene in both cardiac and skeletal muscle cells. Despite the potential of the MEF-2 site sequence as a regulator common to both muscle lineages, there are conflicting reports on the

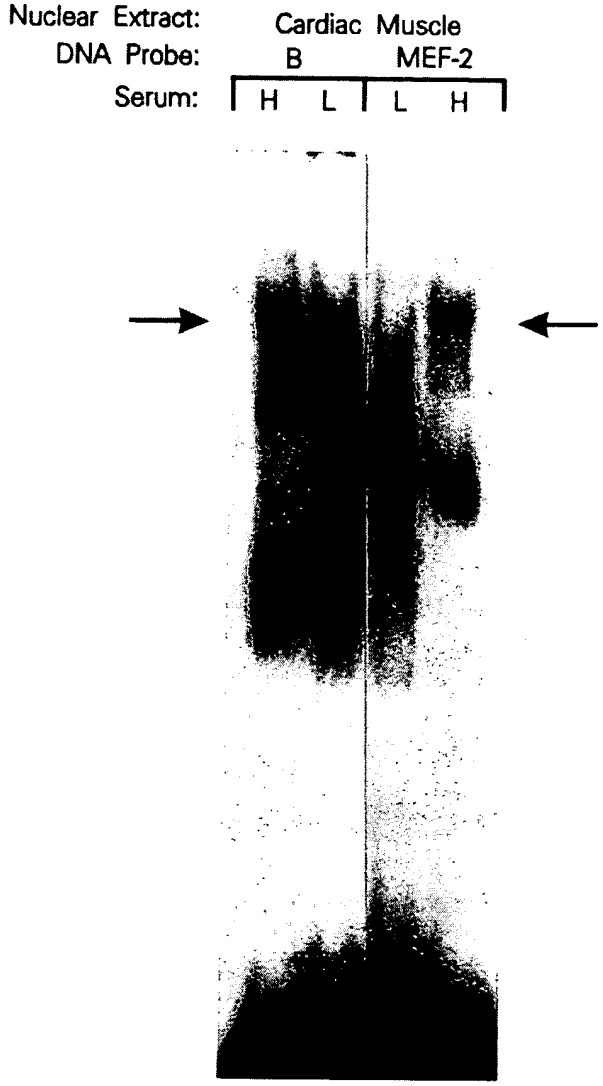


FIG. 9. Serum responsiveness of BBF-1 binding to element B and MEF-2 site DNAs. Cardiac muscle nuclear extracts isolated from primary cells cultured in medium with high levels of serum for 5 days (H) or shifted to low-serum medium after 2 days with high-serum medium (L) were used in a gel mobility shift assay with probes containing element B or MCK MEF-2 site DNAs. Arrows indicate the complex(es) which disappeared in the serum-cultured cells.

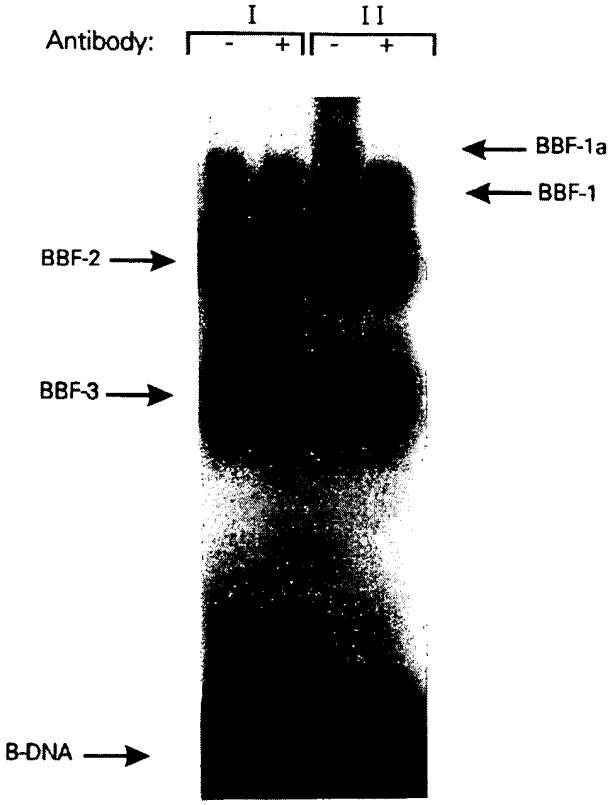


FIG. 10. Comparison of nuclear extracts from cardiac cells cultured for 2 or 5 days. Cardiac nuclear extracts prepared from 5- (I) and 2 (II)-day-culture cells in high-serum medium were used in a gel shift assay with (+) and without (−) preincubation with RSRFC4 antibody. B-DNA was used as a probe. BBF-1a denotes MEF-2 activity (see text).

muscle specificity of protein factors which recognize that sequence. For example, TARP, a non-muscle-specific factor, recognizes the MEF-2 site as well as the AT-rich segments in the brain and muscle creatine kinase promoter and enhancer, respectively (21), and the ubiquitous SRF-related proteins (RSRF) recognize sequences homologous to SRF, TARP, and MEF-2 binding sites (36).

MEF-2 was originally identified as a muscle-specific factor in the skeletal muscle cell line C2 during the transition of the serum-deprived myoblast to myocytes (17). The MEF-2 activity is inhibited by serum in culture medium. Subsequent studies (7, 20, 21) did not confirm the skeletal muscle specificity of protein interactions with the MEF-2 site. It is now believed that both ubiquitous and muscle-specific nuclear factors which bind to the MEF-2 site but with distinct sequence specificities exist (9). Our results here demonstrated that cardiac muscle cells contain at least two MEF-2-like transcription factors, MEF-2 and BBF-1, which both bind to the MEF-2 site in the cardiac MLC-2 promoter (element B), and that one, BBF-1, is cardiac tissue specific and the other, MEF-2, is not. Furthermore, antibodies raised against RSRF recognize MEF-2 but not BBF-1. There is a clear correlation between the appearance of a BBF-1–element B complex and the tissue-specific activity of element B. Taken together, this evidence suggests that cardiac MEF-2 and BBF-1 are members of the MEF-2 family of proteins which have a muscle-specific function but that, unlike the ubiquitous MEF-2, BBF-1 is responsible for cardiac tissue specificity. The potential of element B in cardiac tissue-specific function is also supported by the existence of a 28-bp sequence (HF-1) in rat cardiac MLC-2 (52) which contains within it the core 9-bp-long element B sequence and of a 47-bp segment in the cTnT promoter (22) which shares a distinct homology with element B, both of which are implicated in cardiac tissue-specific transcription.

We have recently reported (41) that the lack of expression of cardiac MLC-2 in skeletal muscle is due to a negative regulatory mechanism which involves an upstream repressor

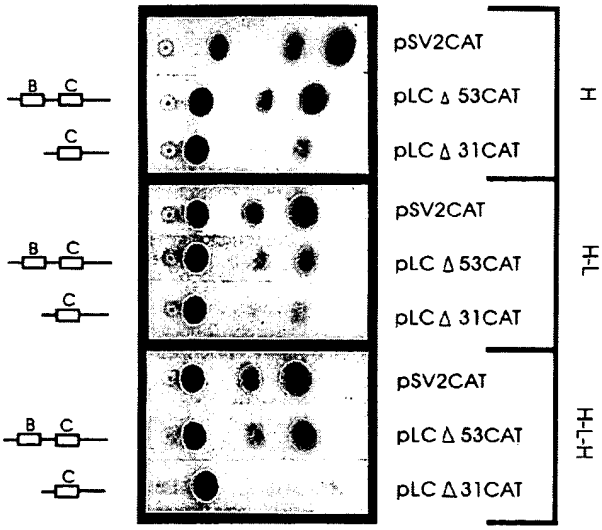


FIG. 11. Serum responsiveness of element B-mediated activation of MLC-2 promoter function. The promoter activity of 5'-deletion mutants containing element C alone (pLCΔ31CAT) and elements B and C (pLCΔ53CAT) was analyzed by transient transfection in cardiac cells grown continuously in high-serum medium for 5 days (H) or shifted to low-serum medium after 2 days in high-serum medium (H-L); some H-L cells were refed with high-serum medium (H-L-H) as described in Materials and Methods.

element, CSS, and its binding proteins, CSFs, present in skeletal muscle. Our data here do not exclude the possibility that element B is not involved in the regulation of cardiac MLC-2 in skeletal muscle. Indeed, the skeletal nuclear proteins recognize element B with sequence specificity. Furthermore, we have observed (unpublished results) that common protein factors might be involved in interactions with these two elements. In early chicken embryonic development, cardiac MLCs are present in both cardiac and skeletal muscle tissues (46), and one could envisage a situation in which the expression of cardiac MLC-2 in early embryonic muscles and its repression in skeletal muscle in later stages of development are functions of a developmental program involving differential syntheses, or activities, of BBFs and CSFs. Conceptually, the developmental changes in MLC-2 expression may be achieved via a combinatorial or preferential use of the positive and negative transcription factors, and we anticipate that such variations in expression and/or utilization of CSFs and BBFs can be experimentally demonstrated. These regulatory sequences and their cognate transcription factors will thus serve as powerful tools for delineating the mechanisms underlying the development and differentiation of cardiac and skeletal muscle lineages (14).

It is well established that the requirements of serum in culture media are different for optimal expression of cardiac tissue- and skeletal muscle-specific genes (6, 8, 13, 14, 24, 35, 40, 42, 43, 45, 47). While reduction of serum levels is required for conversion of the skeletal muscle myoblast to myocytes and activation of skeletal genes, a higher level of serum is needed for optimal expression of cardiac muscle genes (18, 45). It is also recognized that activation of the skeletal myogenic program is dependent upon the expression of MyoD and/or MyoD-related genes (3, 6, 10, 12, 25, 28, 34, 48) following serum withdrawal and through an interaction of MyoD with the target sequence (E-box) in the respective genes. Muscle-specific genes which lack the E-box are presumably regulated through induction of intermediate regulatory factors induced by MyoD or members of the MyoD family. The activation of the MCK gene through MEF-2 is regulated by myogenin, a member of the MyoD family, through a mechanism which requires withdrawal of mitogens from the medium (9), suggesting a pivotal role for MEF-2 in the differentiation pathway of myogenic cells. Mitogenic signals apparently impair regulatory interaction by suppression of MyoD-myogenin expression, and this leads to a block in the skeletal myogenic program.

MyoD family genes are, however, not expressed in cardiac muscle cells. The physiological expression of cardiac muscle genes, which, unlike skeletal genes, are activated by mitogens, might involve factors distinct from the members of the MyoD gene family. No such regulatory factor has so far been isolated. The appearance of BBF-1 due to serum leads to a high level of transcription of cardiac MLC-2 in primary cardiac cells and, conversely, the lack of serum causes its disappearance and a concomitant loss of the promoter activity. We believe that the loss of MLC-2 promoter activity due to serum deprivation is attributable to the disappearance of BBF-1 binding activity. BBF-1 thus appears to be a crucial protein that responds to signals emanating from a cardiac tissue-specific factor(s) functionally analogous to MyoD family proteins. Element B is a potential target site for SRF-related proteins which bind to AT-rich sequences in a number of growth-factor-inducible and muscle-specific genes, including the cardiac MLC-2 gene (36). The serum inducibility of these sites suggests that, like BBF-1, they may also be linked to the extracellular signalling pathway. The notion that accessory proteins participate in SRF interactions has been invoked previously to explain the function of SRF and SRF-related target sites in serum-inducible promoters (36, 37). Such factors might also be involved in functional expression of element B-protein complexes. BBF-1 thus appears to be an important member of the MEF-2 family which would serve as an important tool to advance our understanding of the regulatory mechanisms in cardiogenic differentiation.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01H24315902 and R01H248191.

REFERENCES

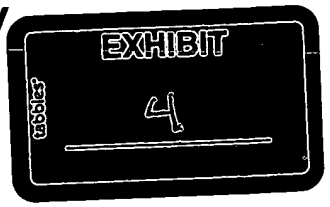
1. **Borras, T., C. A. Peterson, and J. Piatigorsky.** 1988. Evidence for positive and negative regulation in the promoter of the chicken 1-crystallin gene. Dev. Biol. **127**:209–219.
2. **Boxer, L. M., R. Prywes, R. G. Roeder, and L. Kedes.** 1989. The sarcomeric actin CArG-binding factor is indistinguishable from the c-*fos* serum response factor. Mol. Cell. Biol. **9**:515–522.
3. **Braun, T., E. Bober, B. Winter, N. Rosenthal, and H. H. Arnold.** 1990. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. EMBO J. **9**:821–831.
4. **Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H. H. Arnold.** 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. **8**:701–709.
5. **Braun, T., E. Tannich, G. Buschhausen-Denker, and H. H. Arnold.** 1989. Promoter upstream elements of the chicken cardiac myosin light-chain 2-A gene interact with *trans*-acting regulatory factors for muscle-specific transcription. Mol. Cell. Biol. **9**:2513–2525.
6. **Brennan, T. J., D. G. Edmondson, L. Li, and E. N. Olson.** 1991. Transforming growing factor β represses the actions of myogenin through a mechanism independent of DNA binding. Proc.

Natl. Acad. Sci. USA 88:3822–3826.
7. **Buskin, J. N., and S. D. Hauschka.** 1989. Identification of a myocyte nuclear factor that binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. Mol. Cell. Biol. 9:2627–2640.
8. **Clegg, C. H., T. A. Linkhart, B. B. Olwin, and S. D. Hauschka.** 1987. Growth factor control of skeletal muscle differential: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. J. Cell. Biol. 105:949–956.
9. **Cserjesi, P., and E. N. Olson.** 1991. Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. Mol. Cell. Biol. 11: 4854–4862.
10. **Davis, R. L., H. Weintraub, and A. B. Lassar.** 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987–1000.
11. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11:1474–1489.
12. **Edmondson, D. G., and E. N. Olson.** 1989. A gene with homology to the myc similarity region of myoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. 3:628–640.
13. **Eghbali, M., R. Tomek, C. Woods, and B. Bhanbi.** 1991. Cardiac fibroblasts are predisposed to convert into myocyte phenotype: specific effect of transforming growth factor β. Proc. Natl. Acad. Sci. USA 88:795–799.
14. **Emerson, C. D., D. Fischman, B. Nadal-Ginard, and M. A. Q. Siddiqui.** 1986. Molecular biology of muscle development. Alan R. Liss, New York.
15. **Flink, I. L., and E. Morkin.** 1990. Interaction of thyroid receptors with strong and weak cis-acting elements in the human α-myosin heavy chain gene promoter. J. Biol. Chem. 265: 11233–11237.
16. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2:1044–1051.
17. **Gossett, L. A., D. J. Kelvin, E. A. Sternberg, and E. N. Olson.** 1989. A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol. Cell. Biol. 9:5022–5033.
18. **Gupta, M. P., M. Gupta, R. Zak, and V. P. Sukhatme.** 1991. Egr-1, a serum-inducible zinc finger protein, regulates transcription of the rat cardiac α-myosin heavy chain gene. J. Biol. Chem. 266:12813–12816.
19. **Gustafson, T. A., and L. Kedes.** 1989. Identification of multiple proteins that interact with functional regions of the human cardiac α-actin promoter. Mol. Cell. Biol. 9:3269–3283.
20. **Horlick, R. A., and P. A. Benfield.** 1989. The upstream muscle-specific enhancer of the rat muscle creatine kinase gene is composed of multiple elements. Mol. Cell. Biol. 9:2396–2413.
21. **Horlick, R. A., G. M. Hobson, J. H. Patterson, M. T. Mitchell, and P. A. Benfield.** 1990. Brain and muscle creatine kinase genes contain common TA-rich recognition protein-binding regulatory elements. Mol. Cell. Biol. 10:4826–4836.
22. **Iannello, R. C., J. H. Mar, and C. P. Ordahl.** 1991. Characterization of a promoter element required for regulation in myocardial cells. J. Biol. Chem. 266:3309–3316.
23. **Izumo, S., and V. Mahdavi.** 1988. Thyroid hormone receptor a isoforms generated by alternative splicing differentially activate myosin HC gene transcription. Nature (London) 334:539–542.
24. **Kelvin, D. J., G. Simard, H. H. Tai, T. P. Yamaguchi, and J. A. Connolly.** 1989. Growth factors, signal pathway, and the regulation of proliferation and differentiation in BC3H1 muscle cells. I. A. Pertussis toxin-sensitive is involved. J. Cell Biol. 108:159–168.
25. **Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub.** 1989. MyoD is a sequence specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell 58:823–831.
26. **Lee, T.-C., K.-L. Chow, P. Fang, and R. J. Schwartz.** 1991. Activation of skeletal α-actin gene transcription: the cooperative formation of serum response factor-binding complexes over positive *cis*-acting promoter serum response elements displaces a negative-acting nuclear factor enriched in replicating myoblasts and nonmyogenic cells. Mol. Cell. Biol. 11:5090–5100.
27. **Lompre, A., N. Bernardo, and V. Mahdavi.** 1984. Expression of the ventricular α- and β-myosin heavy chain genes is developmentally and hormonally regulated. J. Biol. Chem. 209:6437–6446.
28. **Miner, J. H., and B. Wold.** 1990. Herculin, a fourth member of the myoD family of myogenic regulatory genes. Proc. Natl. Acad. Sci. USA 87:1089–1093.
29. **Minty, A., and L. Kedes.** 1986. Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. Mol. Cell. Biol. 6:2125–2136.
30. **Miwa, T., and L. Kedes.** 1987. Duplicated CArG box domains have positive and mutually dependent regulatory roles in expression of the human α-cardiac actin gene. Mol. Cell. Biol. 7:2803–2813.
31. **Muscat, G. E. O., T. A. Gustafson, and L. Kedes.** 1988. A common factor regulates skeletal and cardiac α-actin gene transcription in muscle. Mol. Cell. Biol. 8:4120–4133.
32. **Nakatsuji, Y., K. Hidaka, S. Tsujino, Y. Yamamoto, T. Mukai, T. Yanagihara, T. Kishimoto, and S. Sakoda.** 1992. A single MEF-2 site is a major positive regulatory element required for transcription of the muscle-specific subunit of the human phosphoglycerate mutase gene in skeletal and cardiac muscle cells. Mol. Cell. Biol. 12:4384–4390.
33. **Navankasattusas, S., H. Zhu, A. V. Garcia, S. M. Evans, and K. R. Chien.** 1992. A ubiquitous factor (HF-1a) and a distinct muscle factor (HF-1b/MEF-2) form an E-box-independent pathway for cardiac muscle gene expression. Mol. Cell. Biol. 12:1469–1479.
34. **Olson, E. D.** 1990. MyoD family: a paradigm for development. Genes Dev. 4:1454–1461.
35. **Olson, E. H., E. Sternberg, G. Spizz, J. S. Hu, and C. Wilcox.** 1986. Regulation of myogenic differentiation by type β transforming growth factor. J. Cell Biol. 103:1799–1805.
36. **Pollack, R., and R. Treisman.** 1991. Human SRF-related proteins DNA-binding properties and potential regulatory targets. Genes Dev. 5:2327–2341.
37. **Prywes, R., A. Dutta, J. A. Cromlish, and R. G. Roeder.** 1988. Phosphorylation of serum response factor, a factor that binds to the serum response elements of the c-fos enhancer. Proc. Natl. Acad. Sci. USA 85:7206–7210.
38. **Qasba, P., E. Lin, M. D. Zhou, A. Kumar, and M. A. Q. Siddiqui.** 1992. A single transcription factor binds to two divergent sequence elements with a common function in cardiac myosin light chain-2 promoter. Mol. Cell. Biol. 12:1107–1116.
39. **Rosenthal, N.** 1989. Muscle cell differentiation. Curr. Opin. Cell Biol. 1:1094–1101.
40. **Schneider, M. D., K. Chow, R. J. Schwartz, and T. G. Parker.** 1991. Growth factor control of myocardial gene transcription. J. Mol. Cell. Cardiol. 23(Suppl. III):S19.
41. **Shen, R., S. K. Goswami, E. Mascareno, A. Kumar, and M. A. Q. Siddiqui.** 1991. Tissue-specific transcription of the cardiac myosin light-chain 2 gene is regulated by an upstream repressor element. Mol. Cell. Biol. 11:1676–1685.
42. **Spizz, G., J. S. Hu, and E. N. Olson.** 1987. Inhibition of myogenic differentiation by fibroblast growth factor of type β transforming growth factor does not require persistent c-myc expression. Dev. Biol. 123:500–507.
43. **Spizz, G., D. Roman, A. Strauss, and E. N. Olson.** 1986. Serum and fibroblast growth factor inhibit myogenic differentiation through a mechanism dependent on protein synthesis and independent of cell proliferation. J. Biol. Chem. 261:9483–9488.
44. **Treisman, R.** 1986. Identification of a protein-binding site that mediates transcription response of the c-fos gene to serum factors. Cell 46:567–574.
45. **Ueno, H., M. B. Perryman, R. Roberts, and M. D. Schneider.** 1988. Differentiation of cardiac myocytes after mitogen with-

drawal exhibits three sequential states of the ventricular growth response. J. Cell Biol. **107**:1911–1918.

46. **Uetsuki, T., Y. Nabeshima, A. Fujisawa-Sehara, and Y.-I. Nabeshima.** 1990. Regulation of the chicken embryonic myosin light-chain (L23) gene: existence of a common regulatory element shared by myosin alkali light-chain genes. Mol. Cell. Biol. **10**:2562–2569.
47. **Waspe, L. E., C. P. Ordahl, and P. C. Simpson.** 1990. The cardiac β-myosin heavy chain isogene is induced selectively in a1-adrenergic receptor-stimulated hypertrophy of cultured rat heart myocytes. J. Clin. Invest. **85**:1206–1214.
48. **Wright, W. E. M., D. A. Sassoon, and V. K. Lin.** 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to myoD. Cell **56**:607–617.
49. **Yu, Y.-T., R. E. Breibart, L. B. Smoot, L. Youngsook, V. Mardavi, and B. Nadal-Ginard.** 1992. Human myocyte enhancer factor 2 comprises a group of tissue restricted MAD box transcription factors. Genes Dev. **6**:1783–1798.
50. **Zarraga, A. M., K. Danishefsky, A. K. Deshpandim, D. Mendola, and M. A. Q. Siddiqui.** 1986. Characterization of 5'-flanking region of heart myosin light chain 2A gene. J. Biol. Chem. **261**:13852–13860.
51. **Zhou, M., Y. Wu, M. Kummar, and M. A. Q. Siddiqui.** 1992. Mechanism of tissue specific transcription: interplay between positive and negative regulatory factors. Gene Expression **2**(2): 127–138.
52. **Zhu, H., A. V. Garcia, R. S. Ross, S. M. Evans, and K. R. Chien.** 1991. A conserved 28-base-pair element (HF-1) in the rat cardiac myosin light-chain-2 gene confers cardiac-specific and α-adrenergic-inducible expression in cultured neonatal rat myocardial cells. Mol. Cell. Biol. **11**:2273–2281.

Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors



Tal Kafri, Ulrike Blömer, Daniel A. Peterson, Fred H. Gage & Inder M. Verma

Successful gene therapy approaches will require efficient gene delivery and sustained expression of the transgene in recipients. A variety of methods, ranging from direct DNA delivery to infection with recombinant viruses containing foreign genes, have been developed, but they all have some major limitations that restrict their utility[1–4]. We have described a human lentiviral (HIV)-based vector that can transduce non-dividing cells *in vitro* and deliver genes *in vivo*[5,6]. With this vector, expression of transgenes in the brain has been detected for more than six months—the longest period tested so far[7]. Because lentiviral vectors are pseudotyped with vesicular stomatitis virus G glycoprotein (VSVG; ref. 8), they can transduce a broad range of tissues and cell types. We now describe the ability of lentiviral vectors to introduce genes directly into liver and muscle. Sustained expression of green fluorescent protein (GFP), used as a surrogate for therapeutic protein, can be observed for more than 22 weeks in the liver. Similar long-term expression (more than eight weeks) was observed in transduced muscle. In contrast, little or no GFP could be detected in liver or muscle transduced with the Moloney murine leukaemia virus (M-MLV), a prototypic retroviral based vector. At a minimum, 3–4% of the total liver tissue was transduced by a single injection of 1–3×10^7 infectious units (I.U.) of recombinant HIV vector. Furthermore, no inflammation or recruitment of lymphocytes could be detected at the site of injection. Animals previously transduced with a lentiviral vector can be efficiently re-infected with lentiviral vectors. Additionally, we show that the requirement for lentiviral accessory proteins to establish efficient transduction *in vivo* is tissue dependent.

To test the ability of lentiviral vectors to deliver genes *in vivo* in liver, we generated high-titre (>3×10^9 I.U./ml) recombinant lentiviruses containing cDNA encoding the red shifted, humanized green fluorescent protein (GFP; ref. 9). Approximately 3×10^7 I.U. of HIV-GFP or MLV-GFP pseudotyped with VSVG protein was injected into the liver parenchyma of adult female nude rats. Fig. 1 shows expression of GFP, detected at the site of injection for at least 22 weeks after injection. There is no reduction in expression of GFP from two weeks (Fig. 1*a*) to 22 weeks (Fig. 1*e*). At higher magnification, propidium-iodide staining marks the nuclei in the section of the liver cells (Fig. 1*b,d,f*) and further shows that GFP is localized in the cytoplasm. No expression could be detected in the livers of animals transduced with MLV-based vec-

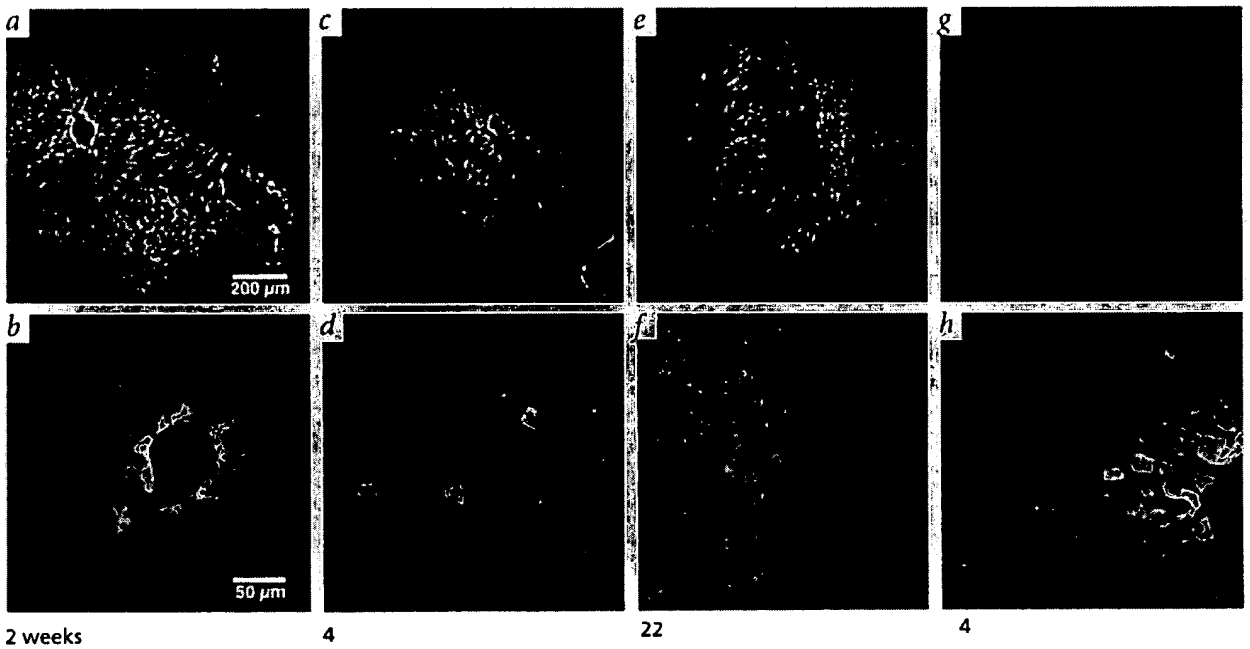


Fig. 1 *In vivo* transduction of rat-liver parenchyma. Nude rat-liver parenchyma was directly injected with 3×10^7 I.U. of either HIV or MLV GFP vectors. Two, four and 22 weeks after HIV-vector injection of nude rats (*a,c,e*) and four weeks after MLV-vector injection (*g*), the transduced livers were sectioned, counterstained with propidium iodide (*b,d,f*) and analysed by confocal microscopy for GFP gene expression. *h*, Data obtained four weeks after transduction of liver of adult Fischer rats. This figure is a composite of sections from six independent experiments. Three independent preparations of viral vectors were used for transductions. One preparation was used to infect two rats for two weeks, another was used to transduce two rats for four weeks and the third preparation was used to infect two nude rats for 22 weeks. The viral vectors used to infect nude rats for 22 weeks was also used to transduce Fischer rats (*h*). The M-MLV recombinant virus was used to infect two nude rats for four weeks; data from one rat are shown (*g*).

Laboratory of Genetics, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037, USA. Correspondence should be addressed to I.M.V. e-mail: verma@salk.edu

Table 1 • Efficiency of transduction

Tissue	Volume sampled (mm^3)	Volume transduced (mm^3)	% volume transduced
Liver	369	332	90
Muscle	245 (±45)	117 (± 9)	49 (±2)

Volume of muscle (2 weeks N=2, 8 weeks N=2) and liver (22 weeks N=1) was determined by point counting according to the Cavalier methods[10]. We examined every tenth 40-μm thick section through a region of the injection (an average of six sections per sample).

tor (Fig. 1*g*). Using point counting[10], we determined the volume of the liver transduced with HIV-GFP vector. In a 369-mm^3 sample, GFP expression could be determined in 332 mm^3, indicating successful transduction of more than 90% of cells at the site of injection (Table 1). Although data shown here were obtained with nude rats, similar results were obtained with wild-type Fischer rats (Fig. 1*h*). We therefore conclude that HIV-based vectors can transduce hepatocytes efficiently at the site of injection (approximately 3–5-mm circumference) and that expression of the transduced gene is sustained.

Muscle is an excellent target for somatic-cell gene therapy[11–13]. It can be accessed easily, and its large mass allows repeat administration of the recombinant vectors. We therefore directly injected $1–3\times10^7$ I.U. of HIV-GFP and MLV-GFP pseudotyped with VSVG protein in the hind-leg muscle of adult Fischer rats. Expression was monitored two, four and eight weeks after injection (Fig. 2*a–d*). Little or no expression was detectable in muscle tissue injected with MLV vectors (Fig. 2 *e,f*). The data obtained with fluorescence were further confirmed by staining with GFP-specific antibodies and indirect immunofluorescence (inset, Fig. 2*a*). We also measured the volume of the muscle tissue transduced at the site of injection. Within a total tissue volume of 245 mm^3 (±45 mm^3), GFP expression could be detected in a volume of 117 mm^3 (±19 mm^3), indicating successful transduction of more than half of the cells (49%±2%) at the site of injection (Table 1).

Because we injected with VSVG-pseudotyped HIV-GFP particles, we were concerned that the host immune system might mount a humoral response to VSVG protein, precluding repeat injection. To explore this possibility, we injected the brains of adult Fischer rats with HIV vector containing the tyrosine hydroxylase (TH) gene (U.B., manuscript in preparation). Fig. 3*a* illustrates the expression of the TH gene in the striatum 175 days after infection. The rats expressing TH were subsequently infected with $1–3\times10^7$ I.U. of HIV-GFP in the hind-leg muscle. Expression of GFP in muscle could be detected two weeks after injection (Fig. 3*b–d*). Thus, it appears that injection in the brain with 3×10^6 I.U. of an HIV vector does not elicit sufficient humoral response to either HIV viral proteins or VSVG protein to prevent successful re-infection with a second recombinant vector at a second site.

No inflammation was observed at the site of injection in the liver or muscle that would be a hallmark for an immune response. To confirm this observation, sections of transduced muscle were examined by immunohistology for the presence of $CD4^+$ or $CD8^+$ lymphocytes and macrophages. Fig. 3*d–f* shows a lack of any infiltrating lymphocytes (depicted in blue) or macrophages in HIV-vector–infected muscle tissues. These types of cells are readily apparent in similar experiments with adenoviral vectors[14]. We extended these observations by injecting 3×10^7 I.U. of HIV-GFP in the left-leg muscle of a rat; after 50 days, another 3×10^7 I.U. of the vector was transduced in the right-leg muscle. The data (Fig. 3*g,h*) show that GFP expression was detected in the muscles of contralateral legs. We therefore conclude that injection with up to 3×10^7 I.U. of VSVG-pseudotyped HIV-based vectors did not elicit sufficient cellular and humoral immune response to inhibit repeat infection.

The HIV vectors used in the experiments described above were generated by three plasmid co-transfections where the viral helper plasmid (pCMVΔR8.2) generates, in addition to gag and pol, the non-essential accessory proteins Vpr, Vpu, Vif, Nef, Tat and Rev[6]. To generate safer vectors that have reduced potential of recombination and that eliminate some of the cytotoxic effects of viral proteins, we constructed viral plasmids unable to synthesize Vpr and Vif proteins. Recombinant HIV-GFP particles without these accessory proteins (deleted packaging constructs; Vpr^-/Vif^-) were generated without compromising the titres of the transducing particles and used to transduce cells *in vivo*. Transduction of neurons in the brain is not affected by lack of Vpr and Vif (Fig. 4*a,b*); in the liver, however, the extent of transduction is substantially reduced (Fig. 4). Thus, it appears that specific HIV auxiliary proteins may be required for maximum transduction of specific tissues.

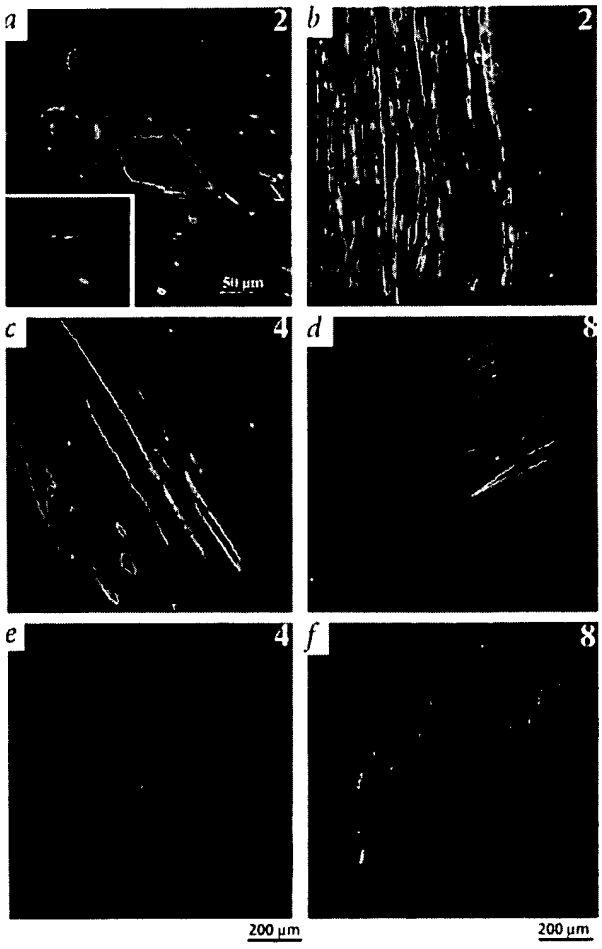


Fig. 2 *In vivo* transduction of rat muscle tissue by lentiviral vector. Fischer rat hind-leg muscles were injected with $1–3\times10^7$ I.U. of either HIV or MLV vectors expressing the GFP gene under the control of the human CMV promoter. Two, four and eight weeks after HIV vector injection (*a–d*), and four and eight weeks after MLV vector injection (as indicated at upper-right-hand side of panel) (*e,f*), the transduced muscles were sectioned, counterstained with propidium iodide and analysed by confocal microscopy for GFP gene expression. The results were further confirmed by immunostaining of the HIV transduced muscle with antibodies to GFP (inset in *a*). This figure is a representative sample of sections from six independent experiments.

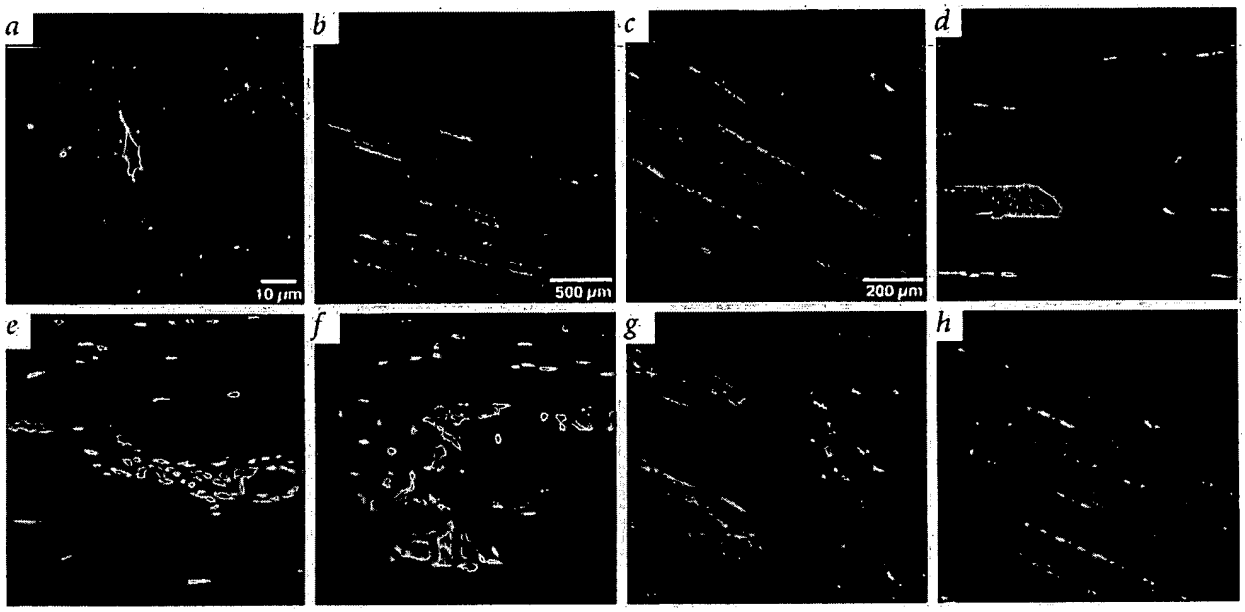
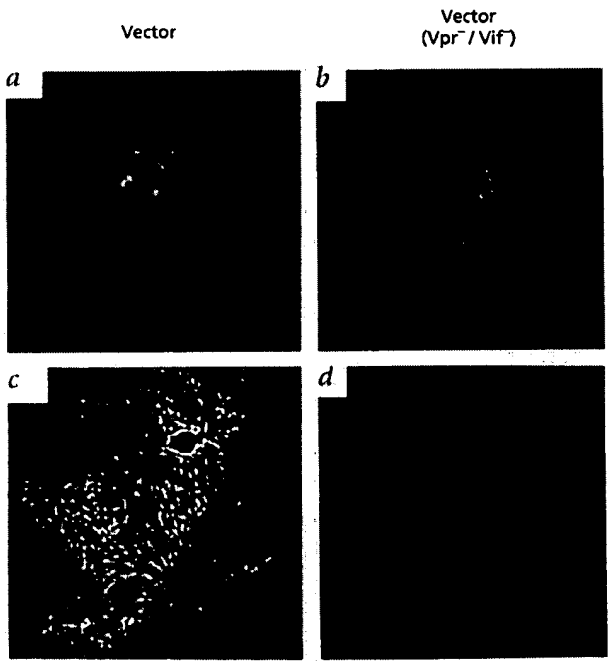


Fig. 3 Immune response after infection with lentiviral vectors. Expression of the tyrosine hydroxylase (*TH*) in Fischer rats (striatum) injected with HIV vector containing the TH gene under the control of the human CMV promoter was confirmed by immunofluorescent staining (green cells) with mouse monoclonal antibodies against *TH* (***a***). HIV-based vector expressing GFP was re-administered into the rat muscle five months after an HIV vector was first injected into the rat brain. Expression of the GFP gene in the transduced muscles was analysed two weeks after injection by confocal microscopy (***b–f***). To examine the immune response against the re-administered vector, we immunofluorescent stained the transduced muscle tissue for invading inflammatory cells CD^+4 or CD^+8 lymphocytes and macrophages (blue staining, *d,e,f,* respectively). Expression of GFP in the left leg of a rat 50 days (***g***) and right leg nine days (***h***) after injection in the same rat at day 51. Two rats were injected in contralateral legs, and data from only one rat are shown.

In this study, we present results that extend the utility of lentiviral vectors for gene transfer *in vivo* to liver and muscle. Furthermore, expression of the transgene can be detected for at least 22 weeks in the liver—the longest period tested so far. Under similar conditions, there is little or no transduction by MLV-based retroviral vectors (Fig. 1*g*). The duration of expression of the transgene in muscle cells has been tested for only eight weeks, but there is no indication that this level will decrease. Measurement of the volumes of muscle and liver tissue transduced showed that infection at the site of injection was very successful, ranging from 50% to 90%. This is a minimal estimate because measurements were made only at the proximal region of the injection site and not at the distant site. Given a total volume of 8,000 mm^3 of liver, we have been able to transduce 3–4% of the whole liver with a single injection of 30 μl of $1–3\times10^7$ I.U. of recombinant vector. The sustained expression of the foreign gene in the context of the lentiviral vectors is surprising, considering that cells transduced with retroviral vectors *in vitro* are subject to transcriptional 'shutoff' *in vivo*[15,16]. Perhaps the LTR in lentiviral vectors is functionally inoperable in the absence of Tat protein; alternatively, direct *in vivo* delivery might not be subject to 'shutoff'.



The lentiviral vectors used did not show inflammation at the site of injection in brain and muscle. In contrast, injection with adenoviral vectors in these tissues recruited $CD8^+$ and $CD4^+$ cells, which eventually led to the elimination of cells producing the transgene product[14,17,18]. In contrast to adenoviral vectors capable of transducing non-dividing cells, the lentiviral vectors could be successfully re-administered (Fig. 3). It should be noted that the titre of the recombinant lentiviruses injected is 3×10^7 I.U., which is considerably lower than that of adenoviral vectors (usually 10^9 I.U.). At higher titres, lentiviral vectors—in particular, with pseudotyped VSVG protein—may elicit a humoral

Fig. 4 The effect of HIV auxiliary proteins on transduction efficiency. Brain (***a,b***) and liver (***c,d***) tissues were transduced by HIV-based vectors expressing the GFP gene under the control of the human CMV promoter. The vectors were generated by using either the ΔR8.2 packaging plasmid containing all the auxiliary viral proteins (*a,c*) or the ΔRnr packaging plasmid from which the *Vpr* and the *Vif* genes had been deleted (*b,d*). The brain was transduced by 3×10^6 I.U., whereas the liver tissue was transduced by 3×10^7 I.U. Two weeks after injection, the transduced tissues were sectioned, propidium-iodine counterstained and analysed by confocal microscopy for GFP gene expression.

response. As lentiviral vectors integrate, however, perhaps re-administration will not be necessary.

To date, high-titre lentiviral vectors have been generated by transient transfection of three plasmids. For utility in human gene therapy, it will be important to generate cell lines that can reproducibly generate helper-free recombinant lentiviruses. Before undertaking the construction of these cell lines, we wanted to examine the role of (and perhaps to eliminate) HIV accessory proteins, particularly cytotoxic ones like Vpr[19–21]. It has been shown that Vpr, a 15-kD virion-associated protein, is required for efficient infection of T-cell lines and primary macrophages by HIV[18–21]. In contrast, we found that the transduction of neurons *in vivo* was undiminished—and may even be enhanced (Fig. 4)—for Vpr^-/Vif^- recombinant virus. Infection of liver parenchymal cells was considerably reduced, however, suggesting that viral accessory proteins may play a tissue-specific role in establishing productive infection. It is possible that neuronal cells contain factor(s) that can compensate for Vpr/Vif function. The HIV vector will allow further investigation into the biology of infection by HIV of different tissues and help to dissect the function of accessory proteins. Finally, the availability of integrating vectors that can deliver genes *in vivo* is a significant advance in protocols for human gene therapy.

Methods

Plasmid construction. The HIV- and MLV-based vectors from which the green fluorescent protein (GFP) is expressed under the control of the human cytomegalovirus promoter pHR′CGFP and pLNCGFP, respectively, were constructed as previously described[6]. The construction of the packaging plasmid ΔR8.2 and the VSVG envelope plasmid pMDG has been described[6]. The *Vpr* and the *Vif* genes were deleted from the ΔR8.2 packaging plasmid by *Eco*RI (partial)/*Nde*I digest, followed by self-ligation that generated the ΔRnr plasmid. This plasmid encodes all the HIV packaging genes except *pVr* and *Vif*. The construction of the pHR′CTH that expresses the tyrosine-hydroxylase gene was carried out as previously described[6]. All the plasmids in this study were produced by a Qiagen maxi-prep kit.

Production and assays of viral vectors. The HIV vectors were produced as described by a transient three-plasmid transfection[5,6]. Briefly, 293T cells were transfected with 7.5 μg of the pMDG envelope plasmid and 15 μg of the various packaging and vector plasmids. Conditioned media were collected 60 h after transfection. The virus was concentrated and dNTPs were treated as previously described[6]. Viral titres on HeLa P4.2 cells were determined by serial dilution and p24 ELISA assay[6]. Absence of replication-competent virus was determined by marker transfer, virus rescue and p24 ELISA assays as described[6]. MLV vectors were produced as previously described[6].

***In vivo* delivery of vector and detection of transgene expression.** All animal procedures were performed in accordance with protocols approved by the Institute Animal Care and Use Committee. Adult female rats (Fischer 344 or nude rats) were anaesthetized by intramuscular injection of ketamine 44 mg/kg, acepromazine 0.75 mg/kg, xylazine 4 mg/kg in 0.9% NaCl i.m. Rat brains were injected in the striatum with 3 μl of the vector ($2–4\times10^6$ I.U). Rat livers were exposed by midline incision of the skin and peritoneum and injected with 30 μl of the vector ($1–3\times10^7$ I.U). Rat hind-leg muscles were injected with 30 μl of the vector ($1–3\times10^7$ I.U). To determine the efficiency of the *in vivo* transduction, the animals were deeply anaesthetized and perfused with 4% cold paraformaldehyde and 0.2% glutaraldehyde intracardially, and the tissues were removed, fixed for 24 h in the perfusion buffer and saturated in 30% sucrose. The fixed tissues were sectioned serially on a freezing microtome (50-μm brain sections, 20- or 40-μm muscle and liver sections) and counterstained with propidium iodide. GFP expression was assayed by fluorescence confocal scanning laser microscopy. Expression of TH and GFP in transduced brain and muscle tissues and the presence of CD^+4, CD^+8, lymphocytes in transduced muscle tissue were determined by immunofluorescence staining. Tissue sections were incubated for 48 h at 4° C in Tris-buffered saline (TBS) with 10% donkey serum and 0.3% Triton X-100 that contained mouse monoclonal antibody to TH (Chemicon, diluted, 1:2,000), rabbit polyclonal antibodies to GFP (Clontech, 1:1,000) or mouse monoclonal antibody to CD4, OX8 and ED1, respectively (Chemicon, 1:2,000). The sections were washed, blocked in TBS with 10% donkey serum and incubated at room temperature with secondary antibodies donkey anti-mouse FITC, donkey anti-rabbit Cy3 and donkey anti-mouse Cy3, respectively. Section analysis was made by confocal scanning laser microscopy (Bio-Rad).

Acknowledgements

We are grateful to L. Ouyang and L. Kitabayoshi for technical assistance. We thank N. Somia, H. Miyoshi and M.L. Gage for critical review of the manuscript. This work was supported by grants from the National Institutes of Health, National Institute for Aging, American Paralysis Association and Hollfelder Foundation. T.K. is supported by a fellowship from Cystic Fibrosis Foundation; U.B. is supported by an NIH grant No. AG08514. We gratefully acknowledge the support of the Frances Berger Foundation. I.M.V. is an American Cancer Society Professor of Molecular Biology.

Received 30 June; accepted 3 October 1997.

1. Mulligan, R.C. The basic science of gene therapy. *Science* **260**, 926–932 (1993).
2. Crystal, R.G. Transfer of genes to humans: early lessons and obstacles to success. *Science* **270**, 404–410 (1995).
3. Leiden, J.M. Gene therapy—promises, pitfalls and prognosis. *N. Engl. J. Med.* **333**, 871–873 (1995).
4. Verma, I.M. & Somia, N. Gene therapy: promises, problems and prospects. *Nature* **389**, 239–242 (1997).
5. Naldini, L., Blomer, U., Gage, F., Trono, D. & Verma, I. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **93**, 11382–11388 (1996).
6. Naldini, L. *et al.* In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**, 263–267 (1996).
7. Blomer, U. *et al.* Highly efficient and sustained gene transfer in adult neurons with a lentiviral vector. *J. Virol.* **71**, 6641–6649 (1997).
8. Burns, J.C., Friedman, T., Driever, W., Burrascano, M. & Yli, J.-K. Vesicular stomatitis virus G protein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90**, 8033–8037 (1993).
9. Miyoshi, H., Takahashi, M., Gage, F.H. & Verma, I.M. Stable and efficient gene transfer into the retina using a lentiviral vector. *Proc. Natl. Acad. Sci. USA* **94**, 10319–10323 (1997).
10. Peterson, D.A., Lucidi-Phillipi, C.A., Eagle, K.L. & Gage, F.H. Perforant path damage results in progressive neuronal death and somal atrophy in layer II of entorhinal cortex and functional impairment with increasing post-damage age. *J. Neurosci.* **14**, 6872–6885 (1994).
11. Barr, E. & Leiden, J.M. Systemic delivery of recombinant proteins by genetically modified myoplasts. *Science* **254**, 1507–1509 (1991).
12. Dhawan, J. *et al.* Systemic delivery of human growth hormone by injection of genetically engineered myoblasts. *Science* **254**, 1509–1512 (1991).
13. Dai, Y., Roman, M., Naviaux, R.K. & Verma, I.M. Gene therapy via primary myoblasts: Long-term expression of factor IX protein following transplantation in vivo. *Proc. Natl. Acad. Sci. USA* **89**, 10892–10895 (1992).
14. Dai, Y. *et al.* Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. *Proc. Natl. Acad. Sci. USA* **92**, 1401–1405 (1995).
15. Palmer, T.D., Rosman, G.J., Osborne, W.R.A. & Miller, A.D. Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc. Natl. Acad. Sci. USA* **88**, 1330–1334 (1991).
16. Scharfmann, R., Axelrod, J.H. & Verma, I.M. Long-term in vivo expression of retrovirus-mediated gene transfer in mouse fibroblast implants. *Proc. Natl. Acad. Sci. USA* **88**, 4626–4630 (1991).
17. Yang, Y., Ertl, J. & Wilson, J.M. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity* **1**, 433–442 (1994).
18. Yang, Y., Li, Q., Ertl, H. & Wilson, J. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol.* **69**, 2004–2015 (1995).
19. Gibbs, J.S., Regier, D.A. & Desrosiers, R.C. Construction and in vitro properties of HIV-1 mutants with deletions in "non-essential" genes. *AIDS Res. Hum. Retroviruses* **10**, 343–350 (1994).
20. Hattori, N. *et al.* The human immunodeficiency virus type 2 *Vpr* genes is essential for productive infection of human macrophages. *Proc. Natl. Acad. Sci. USA* **87**, 8080–8084 (1990).
21. Heinzinger, N.K. *et al.* The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc. Natl. Acad. Sci. USA* **91**, 7311–7315 (1994).

JOURNAL OF VIROLOGY, May 1992, p. 2731–2739
0022-538X/92/052731-09$02.00/0

Vol. 66, No. 5

Human Immunodeficiency Virus Vectors for Inducible Expression of Foreign Genes†

GARY L. BUCHSCHACHER, JR., AND ANTONITO T. PANGANIBAN*

McArdle Laboratory for Cancer Research, University of Wisconsin, 1400 University Avenue, Madison, Wisconsin 53706

Received 13 November 1991/Accepted 3 February 1992

Tat-dependent expression of an endogenous lethal or deleterious foreign gene might be useful for abrogating the production of human immunodeficiency virus (HIV) from cells. This type of HIV-induced cellular killing, as well as other approaches to gene therapy for HIV infection, would be facilitated by simple HIV vectors that express introduced genes in a Tat-inducible manner. As part of studies to examine the feasibility of this concept, we constructed HIV-1 vectors that express the hygromycin B phosphotransferase gene (Hyg^r) in a Tat-dependent manner. Comparison of the efficiency of propagation of each vector indicates that sequences extending into the *gag* open reading frame are necessary in *cis* for efficient vector propagation. Southern blot analysis of genomic DNA isolated from vector-infected cells demonstrated that the vectors were capable of being propagated as expected without gross rearrangements or deletions. A fragment of the influenza A virus hemagglutinin (H5 HA) gene, capable of eliciting antibody and cytotoxic T-cell responses, was used as a marker for further characterization of the vector system. A Tat-dependent vector conferring the H5 HA^+ phenotype was assayed by indirect immunofluorescence, and cells which contained but did not express the H5 HA gene were isolated. The activation of H5 HA expression following HIV infection of Tat^- cells that stably contained but did not express the H5 HA construct was determined to be an efficient process.

A number of advances (for example, see references 37, 43, and 47) make the prospect of using gene therapy as a therapeutic regimen for a variety of human disease processes a realistic goal. Gene therapy could be used either to introduce functional copies of defective genes (for example, see reference 17) or to introduce foreign genes into cells to combat neoplastic or infectious diseases. Replication-defective retroviral vectors are currently the best-characterized method for introducing foreign genes into cells in a relatively controlled manner (reviewed in reference 35). Human trials using retroviral vectors to deliver the tumor necrosis factor or adenosine deaminase genes were recently instituted (3, 8) in efforts to assess their value as therapies for advanced melanoma and adenosine deaminase deficiency, respectively.

AIDS, caused by human immunodeficiency virus (HIV) (7), is a candidate for an infectious disease which might be combated by gene therapy. Because HIV predominantly infects cells of the hematopoietic system, pluri- or multipotent stem cells are potential targets for introduction of a foreign gene which might result in the presence of the gene in functional hematopoietic cells following proliferation and differentiation of the stem cells. Since HIV is a retrovirus, an HIV vector might be useful for delivery of genes of interest. In addition, the nature of specific structural and regulatory genes and of sequences necessary for viral replication suggests strategies for gene therapy that take advantage of HIV-specific *cis*- and *trans*-acting elements.

Relatively complex HIV-1 vectors that express marker genes have been developed to study viral replication (20, 26, 39). Although these could also be used for expression of other foreign genes in cells, it would be advantageous to use a simple vector in which expression is under control of an inducible promoter. The vectors described in this paper express the introduced gene only in the presence of the viral regulatory protein Tat, which is necessary for efficient expression from the HIV long terminal repeat (LTR) (11, 36, 46). Tat is an RNA-binding protein; its target sequence, the TAR region, is a 59-nucleotide sequence present in the newly transcribed R region of the 5′ LTR (42). Although many mechanisms of action have been proposed for Tat, it now appears that Tat acts, at least in part, by preventing premature termination of nascent HIV transcripts (13) in an as yet undetermined manner.

Since Tat is normally found only in cells infected with HIV, this type of vector might provide a means by which foreign genes that interfere with HIV replication could be expressed specifically in HIV-infected cells. This control of gene expression might decrease potential side effects associated with expression of the foreign gene in uninfected cells. Expression of foreign genes under the control of tissue-specific or inducible promoters has been used to ablate specific tissues in transgenic mice (9) and to interfere with viral replication (4, 21). In addition, induced expression of a foreign gene could be used to develop what has been termed intracellular immunity (5) but which, in fact, would more appropriately be viewed as a novel way to deliver therapy.

Examples of types of genes which might be useful in anti-HIV gene therapy include those encoding products that specifically interfere with the HIV life cycle or that are capable of contributing to an induced cytocidal effect. Such a cytocidal effect could result directly from a foreign gene product that is toxic to infected cells or indirectly from a gene product capable of eliciting a specific immune response which destroys infected cells. Genes encoding products to which a person typically has developed immunity (either via natural exposure or vaccination) are candidates for use in inducing a cytocidal effect. Cells that constitutively express hemagglutinin (HA) from one influenza virus strain can be targets for cytotoxic T cells (18). An inducible vector containing a fragment of the H5 HA gene from influenza virus

* Corresponding author.

† This article is dedicated to the memory of Delbert and Leona Buchschacher.

A/Ty/Ont/7732/66 (H5N9) was constructed. This vector was used in these studies as a marker to characterize further the Tat-inducible vector system and its usefulness for delivery of genes expressed from the HIV LTR.

MATERIALS AND METHODS

Vector construction. Standard techniques were used in molecular cloning. The HIV vectors were made in the following manner. To construct pGB102, pAP907 (14), a derivative of the infectious HIV-1 proviral clone pNL4-3 (1), was digested with *Spe*I (in the *gag* open reading frame [ORF]) and *Xba*I (in a polylinker introduced 3′ to the *env* ORF) and the ends were ligated to produce the intermediate clone pGB109. To introduce the hygromycin B phosphotransferase gene (Hyg^r) into pGB109, the plasmid was digested with *Bss*HII and treated with the Klenow fragment. The Hyg^r gene was obtained from pJD214hy (15) by digesting the plasmid with *Acc*I and *Cla*I, treating it with the Klenow fragment, and isolating the appropriate DNA fragment. This fragment was then blunt end ligated into the *Bss*HII-digested pGB109 (regenerating *Bss*HII sites on both sides) to produce the vector pGB102. To construct pGB106, pGB109 was digested with *Xmn*I and *Sma*I and the ends were ligated to produce the intermediate clone pGB105. The Hyg^r gene was isolated from pGB102 by digestion with *Bss*HII and then introduced into the *Bss*HII site of pGB105 to produce the vector pGB106. pGB112 was constructed by digestion of pGB109 with *Bss*HII, treatment with the Klenow fragment, and addition of an *Eco*RI linker to generate the intermediate pGB111. Virginia S. Hinshaw, University of Wisconsin—Madison, provided an H5 HA gene fragment (nucleotides 1 to 1072 [40]) from influenza virus A/Ty/Ont/7732/66 (H5N9) which had been cloned into an SP64-based construct. This was digested with *Hin*dIII and treated with the Klenow fragment, and then an *Eco*RI linker was added. Following digestion with *Eco*RI, the H5 HA fragment was isolated and inserted into the *Eco*RI site of pGB111 to generate the construct pGB112.

Virus production and infections. COS cells were maintained at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. HeLa T4 cells (32) were maintained at 37°C in the same medium supplemented with 7% calf serum, antibiotics, and 800 μg of G418 per ml; HeLa *env-c* cells (provided by Miguel A. Gama Sosa, Dana-Farber Cancer Institute [22]) were maintained in this same medium but without G418. Five micrograms of each DNA was transfected into 10^6 COS cells on 60-mm-diameter tissue culture dishes by the DEAE-dextran procedure (12). On the second day posttransfection, COS cell medium was changed and 4×10^5 HeLa T4 or HeLa *env-c* cells were plated onto 60-mm-diameter dishes for infection the following day. For the cell-free infection, medium was collected from the transfected COS cells and subjected to low-speed centrifugation to remove cell debris. The top portion of the supernatant was removed, and DEAE-dextran was added to a final concentration of 8 μg/ml. One-half milliliter of this supernatant was added to the HeLa cells. Following a 3-h incubation, fresh medium was added. Two days postinfection, selection was initiated for infected cells by fluid changing with medium that contained 200 μg of hygromycin B per ml. Cells were cultured for 7 to 10 days, and the virus titer was determined by fixing and staining the cells with 0.5% crystal violet in 50% methanol and counting resistant colonies.

HeLa cell transfection and selection of Hyg^r cells. For transfection of HeLa T4 or HeLa *env-c* cells, 5×10^5 cells were plated onto 60-mm-diameter dishes. The following day, cells were transfected by the calcium phosphate coprecipitation method (23) with a 15% glycerol shock for 4 min. For single transfections, 11 μg of DNA was used; for cotransfection of a vector and Tat-expressing construct, 8 μg of each DNA was used; for cotransfection of pJD214hy and pGB111 or pGB112, a 20:1 ratio of the two DNAs was used. To determine whether Hyg^r colonies were produced, selection was begun with 200 μg of hygromycin per ml 2 days posttransfection and 7 to 10 days later, if appropriate, resistant colonies were counted as described above. For cells cotransfected with pJD214Hy and pGB111 or pGB112, resistant colonies were pooled or isolated and analyzed by indirect immunofluorescence.

Determination of H5 HA expression. To determine H5 HA expression, indirect immunofluorescence was performed as described previously (51) by using rabbit antiserum prepared to the influenza virus A/Ty/Ont/7732/66 (H5N9), provided by V. S. Hinshaw, at a 1:100 dilution followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody at a 1:40 dilution. When H5 HA and HIV proteins were simultaneously visualized, either FITC- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG antibody (1:32 dilution) was used for H5 HA visualization. For detection of HIV proteins, AIDS patient serum (1:100) and TRITC- or FITC-conjugated goat anti-human antibody (1:40) was used. Filters were used to avoid false-positive signals resulting from leakage of tetramethylrhodamine or fluorescein signals into inappropriate channels. Conjugated antibodies were purchased from Sigma Chemical Co., St. Louis, Mo. To determine H5 HA expression following transfection, 2×10^5 HeLa T4 or HeLa *env-c* cells were plated onto glass coverslips in 35-mm-diameter dishes for transfection as described above. To determine whether the H5 HA gene could be expressed from the HIV-1 vector following infection, virus production and infection were performed as above except that 2×10^5 cells plated on glass coverslips were infected. To determine whether HeLa T4 cells containing the H5 HA-expressing construct could be induced to express H5 HA, GB111- or GB112-containing cells were infected as described above with replication-defective HIV (GB107) pseudotyped with murine amphotropic envelope. Two days posttransfection or postinfection, cells were fixed in ice-cold methanol containing trace acetone.

Southern blotting. Isolated or pooled GB102-infected hygromycin-resistant HeLa *env-c* colonies and pooled GB106-infected hygromycin-resistant colonies were grown, and genomic DNA was isolated by conventional methods. Fifteen micrograms of DNA was used in restriction enzyme digestion. Following electrophoresis through a 0.7% agarose gel, blotting and hybridization were performed as previously described (45) except that salmon sperm or testis DNA was used as the blocking DNA. A double-stranded DNA fragment containing the Hyg^r gene was used as a probe following labeling with [α-^{32}P]dCTP using the random hexamer Prime-a-Gene Labeling System (Promega Biotec, Madison, Wis.). Approximately 7×10^6 cpm of denatured probe per ml was used in the hybridization reaction; incubation was carried out at 65°C for 8 h. The filters were washed with 0.1× SSC (NaCl plus sodium citrate)–0.1% sodium dodecyl sulfate for 2 h at 55°C with agitation, the wash buffer being changed every 20 min. The filters were air dried and exposed to Kodak XAR-5 X-ray film, using a Du Pont Cronex Lightning-Plus intensifying screen.

RESULTS

Development of a Tat-dependent vector system. To construct a simple vector that would be dependent upon Tat for expression of foreign genes, large deletions were made in the infectious proviral clone pNL4-3 (1). Observations with other retroviruses (6, 29, 50) and previous mutational analysis of HIV (2, 10, 30) demonstrate that the untranslated leader region of the genomic-length viral RNA is necessary for efficient packaging. Since the HIV leader region is relatively short, it seemed likely that this encapsidation sequence extends into the *gag* ORF. For this reason, we constructed two vectors (GB102 and GB106; Fig. 1) that contain different lengths of the 5′ *gag* gene. GB106 contains sequences that extend 53 bp into the gene, whereas GB102 contains the first 722 bp of the *gag* ORF. In both vectors the hygromycin B phosphotransferase gene (Hyg^r) gene was placed upstream of the splice donor site so that it would be expressed directly from the LTR. This location of the expressed gene would be likely to prevent expression of the truncated *gag* genes and also results in localization of the putative encapsidation site to near the 3′ end of the genome.

Because Hyg^r expression from these vectors was predicted to be Tat dependent, both HeLa T4 (32) (Tat$^-$) cells and HeLa *env-c* cells (22), which express Tat, were used as target cells for infection. Since HeLa *env-c* cells do not express the major HIV receptor, CD4, a murine amphotropic retrovirus envelope protein was used to pseudotype the vector virus and thereby allow entry into these cells. Virus was produced by transfecting COS cells with vector DNA, a plasmid that expresses all viral proteins necessary for particle production except the envelope glycoprotein (pGB107; Fig. 1), and a plasmid that expresses the envelope glycoprotein of an amphotropic murine leukemia retrovirus (pJD1; Fig. 1). Virus was then harvested, and target cells were infected. The cells were then selected with hygromycin B, and the titer of the vector virus produced was determined by fixing, staining, and counting resistant colonies.

When tested by this assay, GB102 consistently gave a modestly higher titer than GB106 when HeLa *env-c* cells were infected (Table 1). Use of the GB102 vector resulted in a titer of about 100 hygromycin-resistant CFU/ml of supernatant harvested from transfected cells. The GB106 vector resulted in a titer of about 20 CFU/ml. The GB102 vector therefore results in an approximately fivefold higher titer than GB106. No Hyg^r colonies resulted from infection of HeLa T4 cells with GB102 or GB106, demonstrating the Tat dependence of the Hyg^r phenotype following introduction of a single copy of the vector into cells (Table 1). Infection with an HIV-1 vector that expresses the Hyg^r gene from a Tat-independent promoter (GB108; Fig. 1 [20]) resulted in hygromycin-resistant colonies of both cells types. Additional experiments involving introduction of the vector into HeLa T4 cells in the presence or absence of a plasmid that expresses Tat indicated that the level of Tat independence of the vector was approximately 5% (data not shown).

Since GB102 is propagated more efficiently than GB106, it is likely that the 5′ end of the *gag* gene is an ancillary *cis*-acting sequence that facilitates virus propagation. The difference in titer between GB102 and GB106 was not a result of a difference in the ability to express the Hyg^r gene or the ability of the constructs to be transfected; when DNAs were transfected into HeLa *env-c* cells (Tat$^+$) or cotransfected with a Tat-expressing construct into HeLa T4 cells with subsequent hygromycin selection, no difference in the number of Hyg^r colonies was detected. In addition, the fact

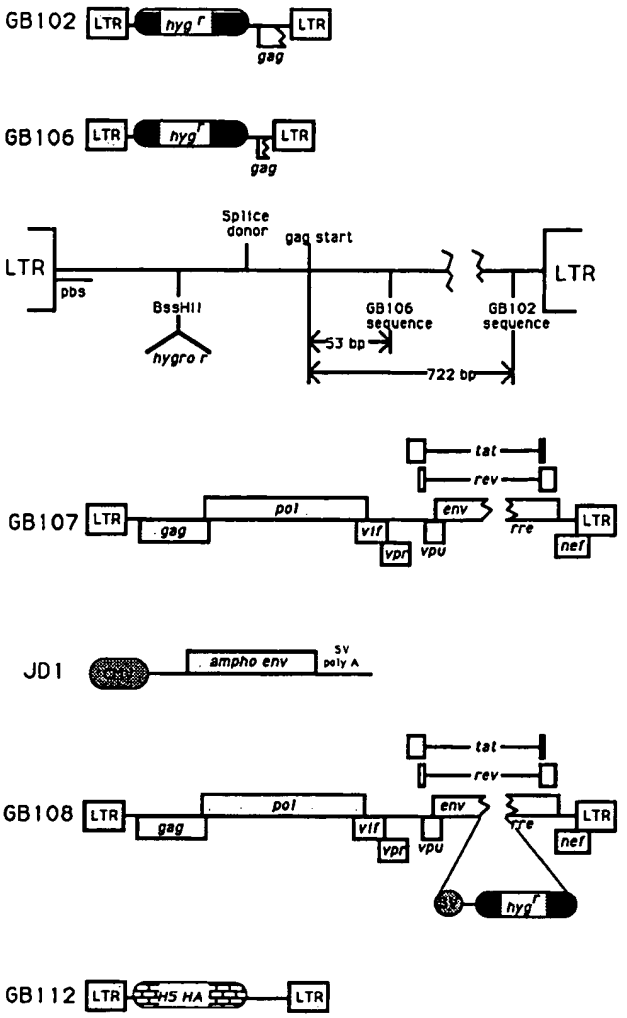


FIG. 1. HIV-1 vectors and expression constructs. Modifications of the infectious molecular clone pNL4-3 were made to generate the HIV vectors and protein-expressing constructs shown. GB102 and GB106 are HIV-1 vectors capable of expressing the Hyg^r gene. Details of their structures are shown below. GB102 and GB106 are identical except for the extent to which *gag* sequences are left intact. GB102 extends 722 bp and GB106 extends 53 bp into the *gag* ORF. The replication-defective helper construct GB107 (20) contains a 426-bp in-frame deletion in the *env* ORF but is capable of expressing other proteins necessary for viral propagation. GB108 (20) is a HIV-1 vector that expresses the Hyg^r gene from the simian virus 40 (SV) early promoter. The murine amphotropic envelope used for pseudotyping HIV-1 core particles is expressed from the cytomeyalovirus (CMV) intermediate-early gene promoter (on the plasmid pJD1 [16]). GB112 is a vector that contains HIV sequences identical to those of GB102; it is capable of expressing a fragment of the influenza virus H5 HA gene directly from the LTR.

that both vectors could be propagated to some degree indicates that placement of the HIV-1 encapsidation region to the 3′ end of the genome still allows RNA packaging, as has been seen with other retroviruses (34).

To determine whether the vectors had been propagated as expected, the structures of proviruses in Hyg^r cells were examined. Genomic DNA from infected, selected HeLa *env-c* cells was prepared from either pooled (GB102 and

TABLE 1. Relative titers of GB102 and GB106 vectors following infection of HeLa *env-c* cells

Expt no.	Hyg^r CFU/ml[a]		
	GB102	GB106	GB102/GB106
1	80	20	4.0
2	100	20	5.0
3	104	24	4.3
4	100	20	5.0
Mean	96 ± 10	21 ± 2	4.6 ± 0.5

[a] The results of four separate experiments are shown. Control values (seen when attempts were made to harvest virus following transfection of COS cells with no DNA, pGB102, pGB106, pGB107, pGB102 plus pGB107, pGB106 plus pGB107, or pGB107 plus pJD1) were all zero. Also, values in experiments using HeLa T4 cells were all zero.

GB106) or isolated (GB102) colonies and used in Southern blot analysis. Following digestion with *Hin*dIII, which cuts once in each proviral LTR, the DNA was electrophoresed, blotted, and probed with a double-stranded DNA fragment of the Hyg^r gene labeled with [α-^{32}P]dCTP. Following autoradiography, bands approximately 2.7 kb in size for GB102 and 2.0 kb for GB106, corresponding to the expected sizes of the respective proviruses, were observed (Fig. 2). Further Southern blot analysis using other restriction enzymes confirmed that the vectors had been propagated without gross rearrangements or deletions. Recombination between vector and defective helper constructs would be expected to result in a Hyg^r-expressing construct with intact HIV ORFs, which would have resulted in the presence of a band of a discrete size. However, no such recombinants were observed. This result indicates that the increased titer of GB102 was not due to recombination resulting from additional *gag* sequences on that vector compared with GB106 but was due to a real difference in ability to be propagated.

We wanted to ensure that the virus was undergoing only a single replication cycle. In addition, we tried to determine whether detectable replication-competent HIV might have been formed by recombination following transfection. Medium was harvested from infected HeLa *env-c* cells, and attempts were made to infect HeLa T4 cells, which are susceptible to infection by virus containing HIV-1 envelope glycoprotein. Also, virus-containing medium from transfected COS cells was used in attempts to infect the HeLa T4 cells. No cytopathic effects were observed in either case. When wild-type HIV produced by transient transfection of COS cells was used to infect these cells, approximately 5,000 syncytium-forming units per ml were observed by a focal immunofluorescence assay.

Tat-dependent expression of a foreign viral gene fragment. We wanted to determine whether a gene on an endogenous Tat-dependent vector could be induced by infection with HIV-1. Moreover, we attempted to construct a vector carrying a gene that could inhibit the generation of progeny virus particles by signalling the immune system to destroy the cell upon infection by HIV. The H5 HA gene of influenza virus A/Ty/Ont/7732/66 (H5N9) is known to elicit murine antibody and cytotoxic T-lymphocyte responses that can result in specific destruction of influenza virus-infected cells (28). Therefore, we inserted a fragment of the H5 HA gene (40) known to contain T- and B-cell antigenic sites (27) in place of the Hyg^r gene of GB102 to generate a vector designated GB112 (Fig. 1). The inserted H5 HA gene encodes the entire HA1 subunit and the first 5 amino acids of

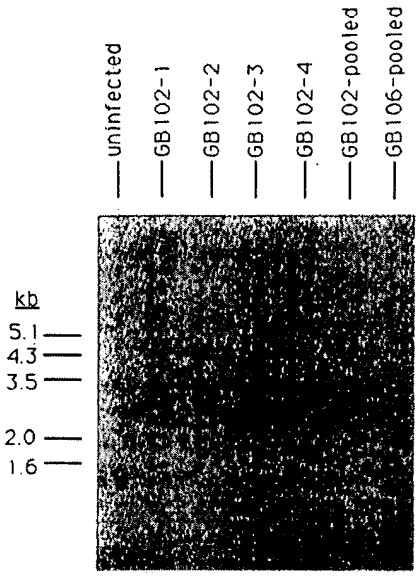


FIG. 2. Southern blot analysis of proviruses from GB102- and GB106-infected HeLa *env-c* cells. Genomic DNA was obtained from isolated (for GB102) or pooled (GB102 or GB106) colonies and digested with *Hin*dIII, which cuts once in each LTR. A ^{32}P-labeled double-stranded DNA fragment of the Hyg^r gene was used as a probe. Fragments of 2.7 kb for GB102 and 2.0 kb for GB106, corresponding to the predicted sizes, were seen, indicating that the vectors had been propagated as expected without gross rearrangements, deletions, or recombination. The structures of the proviruses were confirmed by further Southern blot analysis following digestion with other restriction enzymes.

HA2. In these studies, this construct was used as a marker for expression from the HIV LTR. Eventual evaluation of its use as a functional inducer of cell death via cytotoxic T cells will be dependent upon isolation of an appropriate target cell line which contains the construct. Since at this time the anti-H5 HA cytotoxic T cells which are available are murine major histocompatibility complex restricted (28), a system in which the vector's effect on both HIV infection and the response of cytotoxic T cells can be evaluated is not available.

We first investigated the ability of the H5 HA fragment to be expressed in a Tat-dependent manner. Either pGB112 or a control HIV vector without a foreign gene (pGB111) was transfected in parallel into HeLa *env-c* cells by the calcium phosphate coprecipitation method. In a complementary experiment, an adherent Tat$^-$ cell line (HeLa T4) and a human T-cell line (A3.01) were transfected with various combinations of pGB112 and the Tat-expressing plasmids pSV$_2$tat72 (19) or pGB107. Two days posttransfection, cells were fixed in methanol containing trace acetone and H5 HA expression was determined by indirect immunofluorescence using rabbit antiserum to influenza virus A/Ty/Ont/7732/66. Only cells that had been transfected with both GB112 and Tat-expressing constructs were positive for H5 HA. Examples of H5 HA$^+$ HeLa T4 and HeLa *env-c* foci are shown in Fig. 3A. To determine whether H5 HA expression could be detected following infection with GB112, virus was produced via COS cell transfection (as described above) by using GB111 or GB112 as the vector and was used to infect HeLa *env-c* cells. H5 HA expression, observed 2 days later following indirect immunofluorescence, indicates that the H5 HA gene frag-

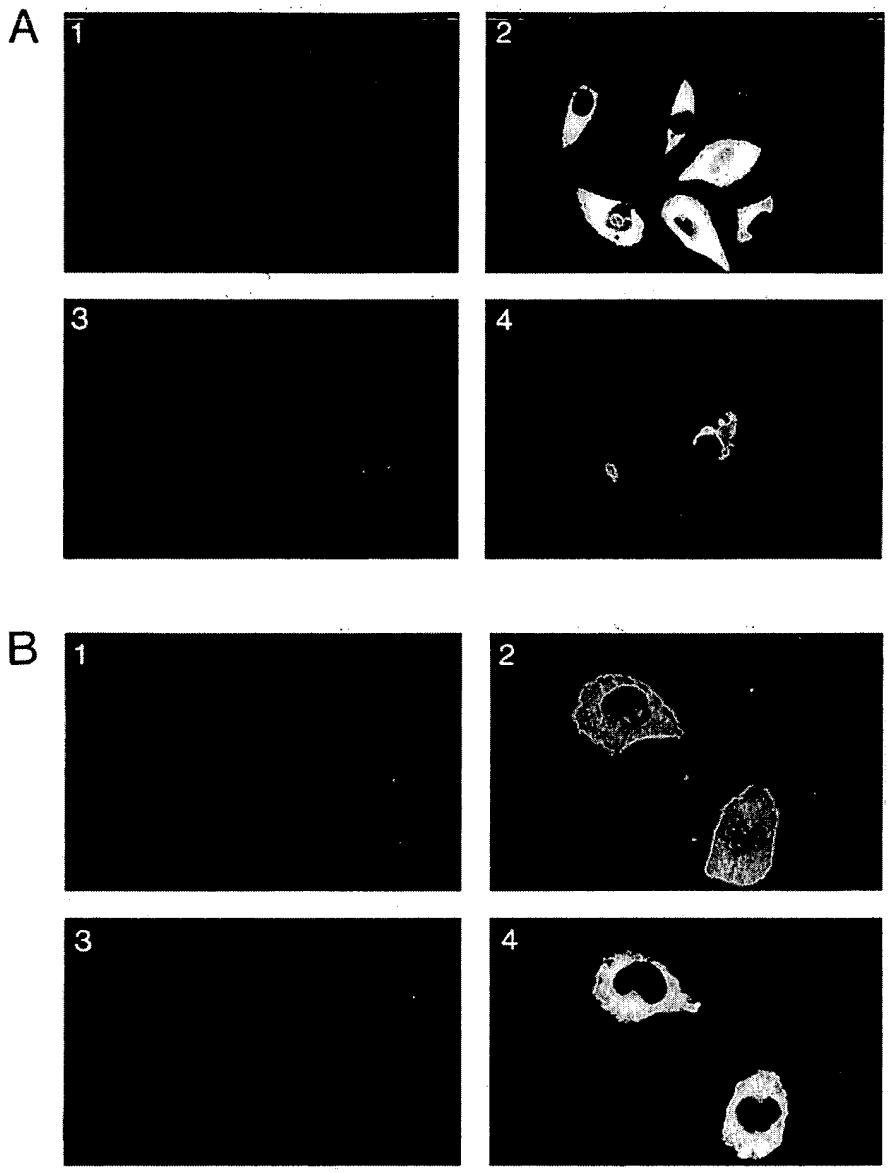


FIG. 3. Immunofluorescence analysis of cells containing H5 HA constructs. (A) Cells (2×10^5) on glass coverslips were transfected by the calcium phosphate coprecipitation method and analyzed for H5 HA expression 2 days later by using rabbit anti-influenza A virus serum and FITC-conjugated goat anti-rabbit IgG antibody following methanol-acetone fixing of the cells. For panels 1 and 3, no positive foci were seen when the entire coverslip was examined. Panel 1, HeLa *env-c* (Tat^+) cells transfected with GB111 (HIV vector with no H5 HA sequences present); panel 2, HeLa *env-c* (Tat^+) transfected with GB112 (expresses H5 HA from HIV LTR); panel 3, HeLa T4 (Tat^-) cells transfected with GB112; panel 4, HeLa T4 cells cotransfected with GB112 and pSV_2tat72. (B) Activation by infection with GB107 (defective HIV construct) of GB112 which is stably maintained but not expressed in HeLa T4 cells (HeLa T4/112 cells) shows coexpression of HIV and H5 HA proteins. Two days following infection with GB107 (approximately 5×10^3 infectious units per ml), HIV protein expression was monitored by using AIDS patient serum and TRITC-conjugated goat anti-human IgG antibody. H5 HA expression was monitored by using rabbit anti-influenza A virus serum and FITC-conjugated goat anti-rabbit IgG antibody. For uninfected cells, no positive H5 HA or HIV foci were seen when the entire coverslip was examined. Panels 1 and 3 and panels 2 and 4 show the same cells photographed with two different filters. Panel 1, HeLa T4/112 uninfected cells, HIV protein expression; panel 2, HeLa T4/112 cells infected with GB107, HIV protein expression; panel 3, HeLa T4/112 uninfected cells, H5 HA protein expression; panel 4, HeLa T4/112 cells infected with GB107, H5 HA expression.

ment is capable of being propagated on the vector and expressed following infection of Tat^+ cells.

For this type of vector system to be useful in abrogation of progeny virus production, it must be possible to introduce the construct into Tat^- cells where it can be stably maintained but not expressed until activated by infection. To determine whether this would be possible, we generated a population of HeLa T4 cells that harbor GB112 (HeLa T4/112 cells) but do not express the H5 HA gene. HeLa T4 cells were cotransfected with either pGB111 or pGB112 and

a construct that expresses the Hyg^r gene from the spleen necrosis virus LTR (pJD214hy) (15). Following hygromycin selection, resistant GB111- or GB112-containing colonies were pooled. These HeLa T4/112 cells were then infected with GB107 (an env-minus, Tat-expressing HIV-1 derivative) that had been pseudotyped with amphotropic envelope glycoprotein. H5 HA expression was seen only in cells that stably contained GB112 and had been infected with the HIV construct. We have also studied the expression of an alternate foreign gene introduced into cells in a similar manner by examining the level of protein present before and after induction by Tat. The results (not shown) indicate that without Tat there is an undetectable level of protein expression when analyzed by immunoprecipitation.

To determine the efficiency of H5 HA gene activation by HIV infection, we isolated and maintained individual HeLa T4/112 clones in culture without hygromycin selection for 4 months. Such clones were identified by screening isolated colonies by transfection with a Tat-expressing plasmid (pSV_2tat72) and subsequent immunofluorescence analysis with anti-HA antiserum. Three HeLa T4/112 clones were expanded and infected with HIV (GB107) particles. Infected cells were identified by using AIDS patient serum and TRITC-conjugated goat anti-human IgG antibody, while cells expressing H5 HA were identified by using rabbit anti-influenza virus antibody and FITC-conjugated goat anti-rabbit IgG antibody. Figure 3B shows a representative example of H5 HA activation. One hundred HIV-positive foci were examined for H5 HA protein expression. The results demonstrated that, on average, 94% of foci expressed both HIV and H5 HA proteins. Approximately equal numbers of the remaining foci were positive for only HIV or H5 HA signals. Variation in the strength of both HIV and H5 HA signals was seen, although there was no correlation between the relative strengths of the two signals. In a complementary experiment, identification of H5 HA and HIV proteins was also done by using FITC-conjugated anti-human and TRITC-conjugated anti-rabbit antibodies.

DISCUSSION

Comparison of two HIV-1 vectors that vary in the putative RNA encapsidation site indicate that the leader region plus approximately 50 nucleotides of the *gag* gene are sufficient for viral nucleic acid propagation. However, the sequences extending further into the *gag* gene significantly increase vector propagation. Although the reason for the difference in vector propagation is not known, the most likely explanation, based on comparison of encapsidation sequences of other retroviruses (6, 29, 50), is that there is a difference in the abilities of the RNAs to be incorporated into virus particles. This interpretation is supported by recent work that indicates that regions of the HIV genome that extend into the *gag* gene are capable of binding purified Gag protein (31). A segment of one RNA sequence that binds to Gag is present in both GB106 and GB102. A second sequence with the ability to bind Gag is present only in GB102. In addition, these sequences have the potential to form complex stem-loop structures which might function in encapsidation and/or RNA-RNA dimer formation. Our observations are consistent with a situation analogous to the *psi* and *psi*$^+$ encapsidation signals of the murine amphotropic retrovirus (6). A vector with *cis*-acting HIV-1 sequences similar to GB106 but with an internal murine retroviral LTR, an internal heterologous polyadenylation site, and an alternative selectable gene which is constitutively expressed has been described elsewhere (41). This vector was successfully propagated, indicating that these sequences are sufficient for propagation.

Since the vector with a longer segment of the 5′ *gag* region was replicated more efficiently than the shorter vector, it would probably be advantageous to use this vector and derivatives to study the *cis*-acting sequences necessary for virus propagation. Further mutational analysis could be used to identify the exact sequences important for encapsidation of HIV and the significance of the potential stem-loop structures in this region. In addition, ways to increase the titer of vector virus produced and development of an alternate system for delivery of HIV vectors are currently being investigated.

It was possible to stably introduce a Tat-responsive vector into cells such that it was maintained but not expressed until infected by exogenous HIV-1 particles. Infection resulted in efficient (about 95%) induction of expression from the vector, on the basis of double-label immunofluorescence with antisera against the virus (AIDS patient serum) and against the Tat-inducible gene product (anti-influenza virus serum). Those cells positive for HIV but negative for H5 HA represent a failure of an incoming virus to activate vector expression to a level detectable by this assay. Those positive for H5 HA but negative for HIV represent either spontaneous activation of the vector or insufficient expression of HIV proteins for detection in the assay. However, since no spontaneous activation of GB112 has been observed in cells that contain the vector but have not been exposed to a Tat-expressing construct, it is probable that the H5 HA-positive, HIV-negative cells represent inapparent infection by HIV.

The construction of a simple Tat-dependent HIV vector might be useful for anti-HIV gene therapy of individuals previously infected by HIV. If an antiviral gene expressed from the HIV LTR can be introduced into a subset of the HIV target cells prior to HIV infection, gene expression might be specifically activated by de novo Tat expression from an incoming virus (Fig. 4). Uninfected cells would not express, or would very inefficiently express, the gene, since Tat is not present in those cells. Since Tat would also promote wild-type virus production, the efficacy of this therapy would depend upon how quickly the infected cell could be destroyed or virus production could be inhibited relative to the time required for progeny virions to be formed. However, if some virus particles are released, this would also lead to propagation of the vector to other, uninfected cells. This would benefit an individual by increasing the number of cells which contain the antiviral gene.

Since this type of strategy involves the expression of genes which are potentially toxic, stringent control of gene expression may be necessary for such an approach to be successful as a therapeutic modality. In the experiments presented here, we measured expression by the production of an easily recognizable phenotype. The significance of background (Tat-independent) expression is unclear and would likely depend on the particular foreign gene on the vector, the cell type infected (unpublished observations and reference 25), or the presence of other factors known to affect LTR expression (for example, see reference 38). It is conceivable that minimal expression of particular genes has no detrimental effect while a similar level of expression of an alternative gene is toxic to the cell. Addition of the *cis* acting Rev-responsive element to the vector, as has been done with other inducible systems (25, 49) using partial HIV-1 LTRs,

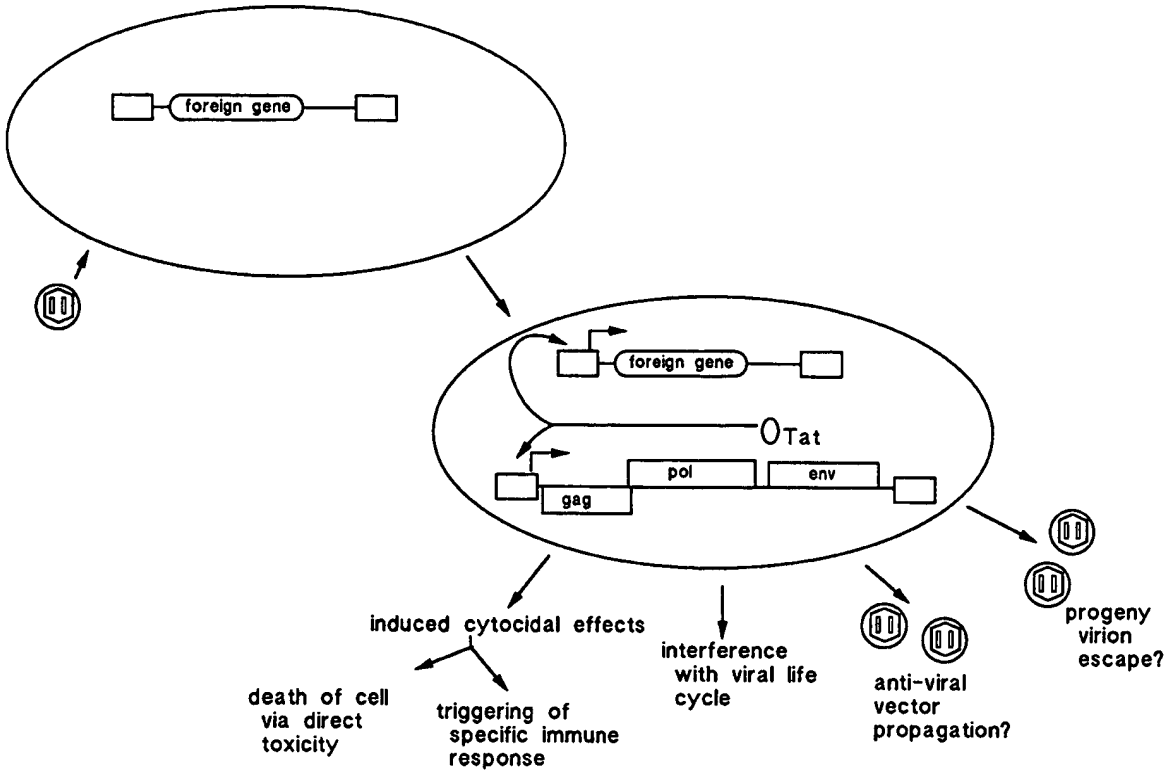


FIG. 4. How inducible gene therapy for HIV infection might work. A construct which contains a foreign gene under control of the HIV LTR is introduced into and stably maintained in a cell susceptible to HIV infection. Because such a cell would not contain the Tat protein necessary for efficient expression from the LTR, the foreign gene would not be expressed to a significant degree. This might decrease potential side effects associated with foreign gene expression. Foreign gene expression is induced by wild-type virus infection. The wild-type virus produces Tat which activates expression from both vector and virus LTRs. The choice of the foreign gene used would determine the effect on the cell. Infected cells could be specifically destroyed by using genes which cause an induced cytocidal effect through either a direct or an indirect mechanism. Alternatively, the release of progeny virus from infected cells might be prevented by using genes which specifically interfere with the HIV life cycle. In either case, the goal of the therapy would be to prevent or slow the spread of HIV within an individual. Because Tat would also be increasing expression of viral proteins, the efficiency of this therapy would depend upon how quickly the infected cell could be destroyed or viral replication could be interfered with relative to the time that it would take for progeny virions to be released. One benefit of using the HIV vector is that any wild-type virus escape which may occur might also be accompanied by vector virus release which could spread to other, uninfected cells, thereby increasing the number of cells containing the antiviral gene.

may offer increased control of gene expression and act as an additional safeguard against inappropriate gene expression.

Types of antiviral genes that might be used to eliminate or slow the spread of HIV include those coding for proteins which directly inhibit the production of infectious HIV. Examples are dominant negative mutants (20, 24, 33, 48) or ribozymes specific for viral RNA sequences (44, 52). For these cases, although the infected cell would remain in the body, virus production would be decreased or prevented. Another class of antiviral genes is those that would directly or indirectly eliminate infected cells through an induced cytocidal effect. Activation of expression of this gene by an incoming virus would induce the cell to commit suicide. Induction of a gene coding for a protein which is toxic to the cell, for example, diphtheria toxin (25) or herpes simplex thymidine kinase (49), would have a direct cytocidal effect. A gene coding for a protein that signals the immune system to destroy the cell (for example, HA) would be an example of an indirect cytocidal effect. Activation of expression of this gene by an incoming virus would result in the production of a protein that the immune system would recognize as foreign. A gene of one of the infectious agents against which human populations are routinely immunized might serve as a suitable foreign gene.

ACKNOWLEDGMENTS

We thank V. S. Hinshaw for helpful discussion, for providing the H5 HA partial clone and rabbit anti-influenza A virus serum, and, along with C. Casella, R. Geraghty, C. Ramsey, and H. M. Temin, for critical reading of the manuscript. We also thank R. Tomar for providing AIDS patient serum, M. A. Gama Sosa for HeLa *env-c* cells, J. Dougherty for the plasmids pJD1 and pJD214hy, and L. Raffini and K. Whelpley for plasmid preparations. The following reagents were obtained from the NIH AIDS Research and Reference Reagent Program: the plasmid pNL4-3 from M. Martin and HeLa T4 cells from R. Axel.

This work was supported by Public Health Service grant CA22443-10 from the NIH and by the Shaw Scholars Fund.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Wiley, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59**:284–291.

2. **Aldovini, A., and R. Young.** 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. J. Virol. **64**:1920–1926.
3. **Antoine, F. S.** 1990. Human gene therapy trial in cancer patients about to begin. J. Natl. Cancer Inst. **82**:1877–1878.
4. **Arnheiter, H., S. Skuntz, M. Noteborn, S. Chang, and E. Meier.** 1990. Transgenic mice with intracellular immunity to influenza virus. Cell **62**:51–61.
5. **Baltimore, D.** 1988. Intracellular immunization. Nature (London) **335**:395–396.
6. **Bender, M. A., T. D. Palmer, R. E. Gelinas, and A. D. Miller.** 1987. Evidence that the packaging signal of Moloney murine leukemia virus extends into the *gag* region. J. Virol. **61**:1639–1646.
7. **Blattner, W., R. C. Gallo, and H. M. Temin.** 1988. HIV causes AIDS. Science **241**:515–516.
8. **Blume, E.** 1990. Gene therapy trial finally begins. J. Natl. Cancer Inst. **82**:1601.
9. **Breitman, M. L., H. Rombola, I. H. Maxwell, G. K. Klintworth, and A. Bernstein.** 1990. Genetic ablation in transgenic mice with an attenuated diphtheria toxin A gene. Mol. Cell. Biol. **10**:474–479.
10. **Clavel, F., and J. M. Orenstein.** 1990. A mutant of human immunodeficiency virus with reduced RNA packaging and abnormal particle morphology. J. Virol. **64**:5230–5234.
11. **Cullen, B. R.** 1986. Trans-activation of human immunodeficiency virus occurs via a bimodal mechanism. Cell **46**:973–982.
12. **Cullen, B. R.** 1988. Use of eukaryotic expression technology in the functional analysis of cloned genes. Methods Enzymol. **152**:684–704.
13. **Cullen, B. R.** 1990. The HIV-1 tat protein: an RNA sequence-specific processivity factor? Cell **63**:655–657.
14. **Delwart, E. L., G. L. Buchschacher, Jr., E. O. Freed, and A. T. Panganiban.** Submitted for publication.
15. **Dougherty, J. P., and H. M. Temin.** 1986. High mutation rate of a spleen necrosis virus-based retrovirus vector. Mol. Cell. Biol. **6**:4387–4395.
16. **Dougherty, J. P., R. Wisniewski, S. Yang, B. W. Rhode, and H. M. Temin.** 1989. New retrovirus helper cells with almost no nucleotide sequence homology to retrovirus vectors. J. Virol. **63**:3209–3212.
17. **Drumm, M. L., H. A. Pope, W. H. Cliff, J. M. Rommens, S. A. Marvin, L. Tsui, F. S. Collins, R. A. Frizzell, and J. M. Wilson.** 1990. Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. Cell **62**:1227–1233.
18. **Eager, K. B., C. J. Hackett, W. U. Gerhard, J. Bennink, L. C. Eisenlohr, J. Yewell, and R. P. Ricciardi.** 1989. Murine cell lines stably expressing the influenza virus hemagglutinin gene introduced by a recombinant retroviral vector are constitutive targets for MHC class I- and class II-restricted T lymphocytes. J. Immunol. **143**:2328–2335.
19. **Frankel, A. D., and C. O. Pabo.** 1988. Cellular uptake of the Tat protein from human immunodeficiency virus. Cell **55**:1189.
20. **Freed, E. O., E. L. Delwart, G. L. Buchschacher, Jr., and A. T. Panganiban.** 1992. A mutation in the human immunodeficiency virus type 1 transmembrane glycoprotein 41 dominantly interferes with fusion and infectivity. Proc. Natl. Acad. Sci. USA **89**:70–74.
21. **Friedman, A. D., S. J. Triezenberg, and S. L. McKnight.** 1988. Expression of a truncated viral trans-activator selectively impedes lytic infection by its cognate virus. Nature (London) **335**:452–454.
22. **Gama Sosa, M. A., R. DeGasperi, F. Fazely, and R. M. Rupert.** 1989. Human cell lines stably expressing HIV env and tat gene products. Biochem. Biophys. Res. Commun. **161**:305–311.
23. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
24. **Green, M., M. Ishino, and P. M. Loewenstein.** 1989. Mutational analysis of HIV-1 tat minimal domain peptides: identification of *trans*-dominant mutants that suppress HIV-LTR-driven gene expression. Cell **58**:215–223.
25. **Harrison, G. S., F. Maxwell, C. J. Long, C. A. Rosen, L. M. Glode, and I. H. Maxwell.** 1991. Activation of a diphtheria toxin A gene by expression of human immunodeficiency virus-1 tat and rev proteins in transfected cells. Human Gene Ther. **2**:53–60.
26. **Helseth, E., M. Kowalski, D. Gabuzda, U. Olshevsky, W. Haseltine, and J. Sodroski.** 1990. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. J. Virol. **64**:2416–2420.
27. **Hioe, C. E., N. Dybdahl-Sissoko, M. Philpott, and V. S. Hinshaw.** 1990. Overlapping cytotoxic T-lymphocyte and B-cell antigenic sites on the influenza virus H5 hemagglutinin. J. Virol. **64**:6246–6251.
28. **Hioe, C. E., and V. S. Hinshaw.** 1989. Induction and activity of class II-restricted, LYT-2+ cytolytic T lymphocytes specific for the influenza H5 hemagglutinin. J. Immunol. **142**:2482–2488.
29. **Katz, R. A., R. W. Terry, and A. M. Skalka.** 1986. A conserved *cis*-acting sequence in the 5′ leader of avian sarcoma virus RNA is required for packaging. J. Virol. **59**:163–167.
30. **Lever, A., H. Gottlinger, W. Haseltine, and J. Sodroski.** 1989. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. J. Virol. **63**:4085–4087.
31. **Luban, J., and S. P. Goff.** 1991. Binding of human immunodeficiency virus type 1 (HIV-1) RNA to recombinant HIV-1 *gag* polyprotein. J. Virol. **65**:3203–3212.
32. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapman, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47**:333–348.
33. **Malim, M. H., S. Bohnlein, J. Hauber, and B. R. Cullen.** 1989. Functional dissection of the HIV-1 rev *trans* activator-derivation of a *trans*-dominant repressor of rev function. Cell **58**:205–214.
34. **Mann, R., and D. Baltimore.** 1985. Varying the position of a retrovirus packaging sequence results in the encapsidation of both unspliced and spliced RNAs. J. Virol. **54**:401–407.
35. **Miller, A. D.** 1990. Progress toward human gene therapy. Blood **76**:271–278.
36. **Muesing, M. A., D. H. Smith, and D. J. Capon.** 1987. Regulation of mRNA accumulation by a human immunodeficiency virus transactivator protein. Cell **48**:691–701.
37. **Nabel, E. G., G. Plautz, and G. J. Nabel.** 1990. Site-specific gene expression in vivo by direct gene transfer into the arterial wall. Science **249**:1285–1288.
38. **Nabel, G. J., S. A. Rice, D. M. Knipe, and D. Baltimore.** 1988. Alternative mechanisms for activation of human immunodeficiency virus enhancer in T cells. Science **239**:1299–1301.
39. **Page, K. A., N. R. Landau, and D. R. Littman.** 1990. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. J. Virol. **64**:5270–5276.
40. **Philpott, M., C. Hioe, M. Sheerar, and V. S. Hinshaw.** 1990. Hemagglutinin mutations related to attenuation and altered cell tropism of a virulent avian influenza A virus. J. Virol. **64**:2941–2947.
41. **Poznansky, M., A. Lever, L. Bergeron, W. Haseltine, and J. Sodroski.** 1991. Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. J. Virol. **65**:532–536.
42. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell **41**:813–823.
43. **Rosenberg, S. A., P. Aebersold, K. Cornetta, A. Kasid, R. A. Morgan, R. Moen, E. M. Karson, M. T. Lotze, J. C. Yang, et al.** 1990. Gene transfer into humans—immunotherapy of patients with advanced melanoma using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N. Engl. J. Med. **323**:570–578.
44. **Sarver, N., E. M. Cantin, P. S. Chang, J. A. Zaia, P. A. Ladne, D. A. Stephens, and J. J. Rossi.** 1990. Ribozymes as potential anti-HIV-1 therapeutic agents. Science **247**:1222–1225.

45. **Schedl, T., and W. F. Dove.** 1982. Mendelian analysis of the organization of actin sequences in *Physarum polycephalum*. J. Mol. Biol. **160**:41–57.
46. **Sodroski, J., C. Rosen, F. Wong-Staal, S. Z. Salahuddin, M. Popovic, S. Arya, R. C. Gallo, and W. A. Haseltine.** 1985. Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. Science **227**:171–173.
47. **Spangrude, G. J., S. Heimfeld, and I. L. Weissman.** 1988. Purification and characterization of mouse hematopoietic stem cells. Science **241**:58–62.
48. **Trono, D., M. B. Feinberg, and D. Baltimore.** 1989. HIV-1 Gag mutants can dominantly interfere with the replication of the wild-type virus. Cell **59**:113–120.
49. **Venkatesh, L. K., M. Q. Arens, T. Subramanian, and G. Chinnadurai.** 1990. Selective induction of toxicity to human cells expressing human immunodeficiency virus type 1 Tat by a conditionally cytotoxic adenovirus vector. Proc. Natl. Acad. Sci. USA **87**:8746–8750.
50. **Watanabe, S., and H. M. Temin.** 1982. Encapsidation sequences for spleen necrosis virus, an avian retrovirus, are between the 5′ long terminal repeat and the start of the gag gene. Proc. Natl. Acad. Sci. USA **79**:5986–5990.
51. **Weaver, T. A., and A. T. Panganiban.** 1990. N myristoylation of the spleen necrosis virus matrix protein is required for correct association of the Gag polyprotein with intracellular membranes and for particle formation. J. Virol. **64**:3995–4001.
52. **Weerasinghe, M., S. E. Liem, S. Asad, S. E. Read, and S. Joshi.** 1991. Resistance to human immunodeficiency virus type 1 (HIV-1) infection in human $CD4^+$ lymphocyte-derived cell lines conferred by using retroviral vectors expressing an HIV-1 RNA-specific ribozyme. J. Virol. **65**:5531–5534.

JOURNAL OF VIROLOGY, Sept. 1994, p. 6047–6051
0022-538X/94/$04.00+0

Vol. 68, No. 9

NOTES

A Human Immunodeficiency Virus Type 1 (HIV-1)-Based Retroviral Vector System Utilizing Stable HIV-1 Packaging Cell Lines

RICHARD CARROLL,[1]* JEN-TSUN LIN,[1] EMELYN JOY DACQUEL,[1] JOSEPH D. MOSCA,[1] DONALD S. BURKE,[2] AND DANIEL C. ST. LOUIS[1]

Research Laboratory, Henry M. Jackson Foundation for the Advancement of Military Medicine,[1] and Walter Reed Army Institute of Research,[2] Rockville, Maryland 20850

Received 16 February 1994/Accepted 20 May 1994

We have constructed stable human immunodeficiency virus (HIV) packaging cell lines that when transfected with an HIV-based retroviral vector produce packaged vectors capable of transducing susceptible CD4$^+$ cells. This HIV-1-based retroviral vector system has the potential for providing targeted delivery and regulated expression of immunogens or antiviral agents in CD4$^+$ cells.

The ability of retroviral vectors to integrate into the genome of target cells renders them effective gene transfer vehicles (7). Retroviral vector systems typically consist of two components: a packaging cell line and a replication-defective vector. The packaging cell line synthesizes the viral structural proteins required for assembly of infectious virus-like particles. The vector contains the *cis*-acting signals required for incorporation of the vector into virions, initiation of reverse transcription, and insertion of vector DNA into the host genome.

A human immunodeficiency virus type 1 (HIV-1)-based retroviral vector system represents a potentially attractive approach to combating HIV-1 infection in susceptible cells. HIV-1 virus-like particles containing the HIV envelope glycoprotein gp120^{Env} can be selectively targeted to CD4$^+$ cells (2). Moreover, HIV-based vector gene expression can be rendered dependent on *trans*-acting signals encoded by HIV (reviewed in reference 5). We report the development of an HIV-based retroviral vector system employing a stable HIV packaging cell line. When transfected with an HIV-derived retroviral vector, this cell line produces packaged HIV vectors that transduce CD4$^+$ cells. Furthermore, vector gene expression in the transduced cells is responsive to HIV regulatory proteins.

Construction and characterization of HIV-based packaging cell lines. All HIV-1 sequences were derived from the molecular clone HXBc2 (8), and nucleotides are numbered as described previously (24). The packaging cell expression vector pHIVΔψHYG (Fig. 1) was constructed by generating a previously described (1) 21-nucleotide (nt) deletion (nt 294 to 314) in the HXBc2 packaging (ψ) site (18) by site-directed mutagenesis using a Muta-Gene kit (Bio-Rad). In addition, the HXBc2 *nef* gene and 3′ long terminal repeat (LTR) were replaced with the hygromycin phosphotransferase (Hyg^r) gene and a synthetic polyadenylation signal. The Hyg^r-encoding plasmid pHph+1 (Boehringer Mannheim) was modified by inserting a synthetic polyadenylation signal (AATAAA) and a *Cla*I site at the Hyg^r 3′ terminus. *Nco*I sites were introduced at both the Hyg^r translation initiation codon and the HXBc2 *nef* initiation codon (nt 8422 to 8424). The altered HXBc2 and Hyg^r coding sequences were inserted into a pUC19-derived plasmid backbone, yielding pHIVΔψHYG.

Vero cells were transfected with pHIVΔψHYG by the calcium phosphate procedure (9) and cultured in the presence of 200 μg of hygromycin B (Sigma) per ml. Supernatant from hygromycin B-resistant clones was assayed for p24^{gag} production by using a commercially available kit (Coulter), and high-producer clones were subcloned. Two subclones that produced the highest levels of p24^{Gag} antigen (D3.2 and B4.7; Table 1) were assayed for supernatant reverse transcriptase activity as described previously (15). Also, virus-like particles pelleted from packaging cell supernatant were analyzed for the presence of full-length HIV RNA by reverse transcription PCR using primers from the HIV-1 *gag* coding region as described previously (20).

As a positive control in these characterization studies, Vero cells were transfected with pProNeo (Fig. 1), an infectious HXBc2-derived proviral clone carrying the neomycin phosphotransferase (Neo^r) gene in place of the *nef* gene (22). Transfected cells were selected in the presence of 600 μg of G418 (Geneticin; Gibco-BRL) per ml. The G418-resistant subclone producing the highest levels of p24^{gag} antigen (V1.8) was characterized further.

While p24^{Gag} antigen production by clones D3.2 and B4.7 was roughly comparable to the values obtained in V1.8 cells, reverse transcriptase activities in the supernatants of D3.2 and B4.7 cells were approximately 20- and 60-fold lower, respectively, than the level observed in V1.8 cell supernatant (Table 1). Virus-like particles produced by the D3.2 and B4.7 packaging cell lines contained full-length viral RNA, but at only approximately 5% of the level observed in particles produced by V1.8 cells (Table 1). This low level of packaged RNA is consistent with previous reports examining encapsidation of ψ-minus transcripts (1, 4, 13).

Expression of HIV structural proteins in the stably transfected cell lines was examined by radioimmunoprecipitation analysis. The cells were labelled overnight with [^{35}S]methionine. Cell lysates were adjusted to contain equal amounts of trichloroacetic acid-precipitable radioactivity, and HIV structural proteins were precipitated with either polyclonal antisera directed against p24^{Gag} or gp120^{Env} (both obtained from American Biotechnologies) or AIDS patient antisera. The

* Corresponding author. Mailing address: Henry M. Jackson Foundation for the Advancement of Military Medicine, 1600 E. Gude Dr., Rockville, MD 20850. Phone: (301) 217-9410. Fax: (301) 762-7460.

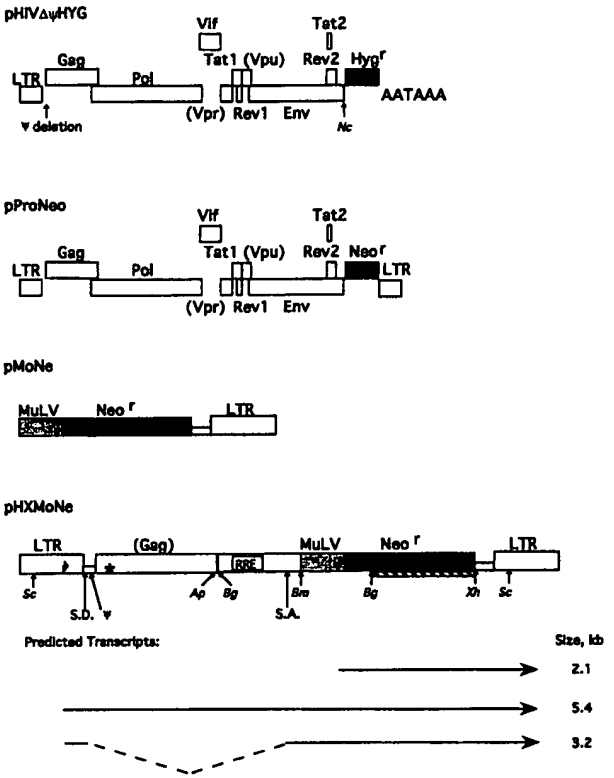


FIG. 1. Structures of packaging cell constructs and retroviral vectors. Open boxes represent HIV-derived structural regions, filled boxes denote genes encoding selectable markers (Neo^r and Hyg^r), and the shaded box represents the MoMuLV promoter. Parentheses indicate unexpressed open reading frames. In the HIV-1 proviral clone HXBc2, the *vpu* open reading frame is not expressed and the *vpr* open reading frame is truncated (24). In pHIVΔψHYG, the positions of the ψ site deletion and the synthetic polyadenylation signal (AATAAA) are indicated. In pHXMoNe, the transcription start sites are indicated by horizontal arrows (predicted transcripts are shown below the construct). The positions of the major splice donor (S.D.), ψ site, and RRE are indicated. The frameshift mutation introduced into the pHXMoNe *gag* coding sequences is depicted by an asterisk. S.A. denotes three splice acceptor sites clustered within a 30-nt region (29). The horizontal hatched bar depicts a 1-kb *Bgl*II-*Xho*I Neo^r gene fragment used as a probe in hybridization analyses. Abbreviations for relevant restriction enzyme sites: *Nc*, *Nco*I; *Sc*, *Sca*I; *Ap*, *Apa*I; *Bg*, *Bgl*II; *Bm*, *Bam*HI; *Xh*, *Xho*I.

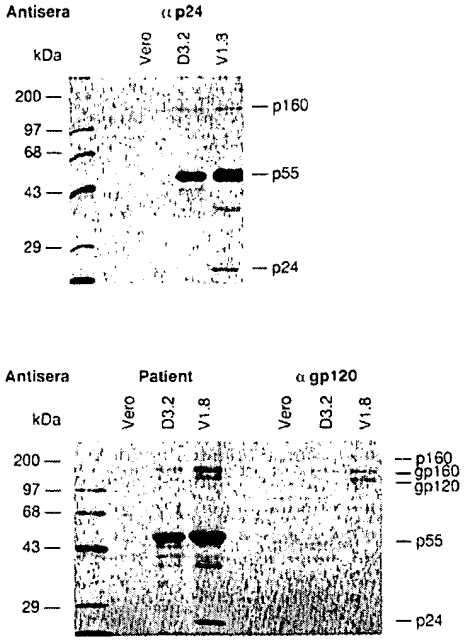


FIG. 2. Radioimmunoprecipitation analysis of stable HIV packaging cell lines. Lysates of [^{35}S]methionine-labelled D3.2 packaging cells, Vero cells, and the chronic virus-producing cell line V1.8 were incubated with AIDS patient antisera or polyclonal antisera directed against the HIV-1 envelope glycoprotein (αgp120) or capsid protein (αp24). Precipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Molecular sizes are indicated, as are the positions of viral precursor and processed proteins.

results obtained for the D3.2 cell line are shown in Fig. 2. HIV precursor polyproteins p55^{Gag}, p160$^{Gag/Pol}$, and gp160^{env} were readily detected in the D3.2 line, and processing of the gp160^{Env} precursor to mature gp120^{Env} was observed. However, processed p24^{Gag} capsid protein was barely detectable in D3.2 cells, suggesting that p55^{Gag} processing was impaired in this line.

Production of transducing particles. The ability of the HIV packaging cell lines to generate transducing particles was examined by using the HIV-derived vector pHXMoNe (Fig. 1). The 5′ portion of pHXMoNe consists of a 3-kb *Xba*I-*Apa*I HXBc2 fragment ligated to an 850-nt *Bgl*II-*Bam*HI HXBc2 fragment. The *Xba*I-*Apa*I fragment contains approximately 1 kb of cellular flanking DNA, the 5′ LTR, untranslated leader sequences, and a fragment of *gag* coding sequences (ending at nt 1555). A frameshift mutation introduced at the unique *Cla*I site (nt 375 to 380) terminates *gag* translation after 21 codons. The *Bgl*II-*Bam*HI fragment contains the Rev response element (RRE [6, 26]) as well as the splice acceptors located upstream of the second *tat* and *rev* coding exons and the *nef* open reading frame (29). A 1.7-kb fragment containing the Moloney murine leukemia virus (MoMuLV) promoter (33) and Neo^r gene was inserted downstream of the *Bgl*II-*Bam*HI fragment. The polyadenylation/termination signal present in the MoMuLV LTR is absent from this fragment. The 3′ terminus of pHXMoNe consists of a portion of the HXBc2 *nef* gene (beginning at the *Xho*I site at nt 8442 to 8447), the polypurine tract, and the 3′ viral LTR.

The construct pMoNe (Fig. 1) was derived from pHXMoNe to serve as a control in transduction assays. It contains the

TABLE 1. Characterization of particles produced by packaging cell lines[a]

Cell line	p24 antigen (ng/10^6 cells/ml)	Reverse transcriptase activity (cpm/10^6 cells/ml)	Genomic RNA in viral particles[b]
V1.8	80	1.2×10^5	100.0
D3.2	68	5.2×10^3	6.0
B4.7	40	2.1×10^3	3.8
Vero	0	0	NA

[a] Values represent means of three separate experiments.
[b] Cell supernatants were normalized for p24^{Gag} content, and genomic RNA content of pelleted particles was determined by reverse transcription PCR analysis. RNA levels are expressed relative to the level detected in particles pelleted from the V1.8 line. NA, not applicable.

TABLE 2. G418 resistance in SupT1 cells transduced with HIV vectors

Cell line	Vector	Trial	Titer (transducing particles/ml)[a]
D3.2	pHXMoNe	1	10^1
		2	10^2
		3	10^1
		4	10^1
B4.7	pHXMoNe	1	10^0
		2	10^0
		3	0
D3.2/B4.7[b]	pMoNe	1	0
		2	0
		3	0
Vero	pHXMoNe	1	0
		2	0
		3	0

[a] Determined by limiting dilution analysis. Transduction was detected by the appearance of G418-resistant cells. Indicated titers represent minimum values.
[b] Two trials used pMoNe-transfected D3.2 cells, while the other used pMoNe-transfected B4.7 cells.

MoMuLV promoter, the Neo^r gene, and the 3′ HIV LTR, but it lacks the 5′ HIV LTR, untranslated leader, *gag*, and RRE regions.

Packaging cell lines D3.2 and B4.7 were transfected with pHXMoNe and selected in 600 μg of G418 per ml. G418-resistant cells were pooled, and 2×10^6 SupT1 cells were infected with 10-fold serial dilutions of filtered (0.45-μm-pore-size filter) pooled packaging cell supernatant. The SupT1 cells were placed under G418 selection and monitored for the appearance of G418-resistant cells. Transducing particle titers were defined as the lowest dilution of filtered supernatant that yielded resistant cells. It should be emphasized that these values represent minimum titers.

Transducing particle titers from pools of transfected B4.7 cells were extremely low (Table 2). In two cases, 10^0 particles per ml were produced, while in a third instance, no transducing particles were detected. Slightly higher transducing particle titers were obtained from pools of transfected D3.2 cells (Table 2). In one instance, 10^2 transducing particles per ml were produced. In three other experiments, 10^1 transducing particles per ml were generated.

The low number of transducing particles produced by pooled pHXMoNe-transfected packaging cells suggested that a minority of the cells in these populations could have produced high titers of transducing particles, events obscured by a large background of nonproducer cells. To address this issue, clonal pHXMoNe-transfected packaging cells were established and assayed for transducing particle production. While a high percentage of clonal lines produced transducing particles, none had titers higher than 10^2 particles per ml (data not shown), suggesting that the existence of high-titer packaging cell clones, while possible, was unlikely.

To exclude nonretroviral mechanisms of Neo^r gene transfer to SupT1 cells, Vero cells were transfected with pHXMoNe. The resulting cell pools contained functional vectors but were unable to generate virus-like particles. Similarly, D3.2 and B4.7 cells were transfected with pMoNe, yielding virus-like particle-producing cells containing vectors that could not be encapsidated or reverse transcribed. No transducing particles were produced by either type of cell population (Table 2), demonstrating that transduction requires both functional vectors and virus-like particle-producing cells.

Replication-competent retroviruses are frequent contaminants of retroviral vector preparations, most frequently arising through recombination between viral sequences in the packaging cell line and the vector (21). The presence of replication-competent retroviruses in supernatant from pHXMoNe-transfected packaging cells was assayed by the following method. SupT1 cells were infected with 1 ml of filtered supernatant and cultured without selection for 30 days, during which time they were monitored for syncytium formation. The 30-day culture supernatant was used to infect fresh SupT1 cells, which were assayed for $p24^{Gag}$ antigen expression 5 days later. $p24^{Gag}$ antigen levels were indistinguishable from background for all transfected packaging cell populations examined (data not shown). This assay, when performed with an HIV_{IIIB} stock, had a sensitivity threshold of 0.1 50% tissue culture infective dose per ml (data not shown). These results demonstrate that under the culture conditions used, no replication-competent retrovirus was detectable in transducing particle-producing cell lines.

Characterization of transduced SupT1 cells. SupT1 cells transduced by supernatant from pools of pHXMoNe-transfected packaging cells were examined for the presence of pHXMoNe vector DNA by Southern blotting (28). *Sca*I-digested genomic DNA was hybridized with a 1-kb probe derived from the Neo^r region of pHXMoNe. The Neo^r probe hybridizes to a 4.8-kb internal *Sca*I fragment (Fig. 1). The predicted fragment was recognized in all transduced SupT1 populations, suggesting that vector integration occurred without gross structural rearrangement (Fig. 3A). While this analysis of integration is indirect, the sequences have been retained in the cells for more than 4 months (data not shown), which strongly suggests that they have been stably transferred.

HIV vector-directed gene expression in SupT1 cells transduced by supernatant from pools of pHXMoNe-transfected packaging cells was examined by Northern (RNA) blotting analysis (28). Predicted vector transcripts are shown in Fig. 1. Total RNA was isolated by using RNAzol B (Tel-Test) and hybridized with the 1-kb Neo^r probe (Fig. 3B). The Neo^r probe hybridized strongly to a 2.1-kb band, consistent with the size predicted for MoMuLV transcripts. The Neo^r probe also recognized a less abundant transcript of approximately 3.2 kb. Spliced HIV promoter transcripts (utilizing the splice acceptors downstream of the RRE [Fig. 1]) are predicted to be 3.2 kb in size. However, the Neo^r probe did not detect the predicted 5.4-kb unspliced HIV promoter transcript.

The ability of HIV-1 regulatory gene products to modulate HIV vector-directed gene expression in transduced SupT1 cells was examined by using the *tat* expression plasmid pIIIEx-Tat (32) and the *rev* expression plasmid pCMVRev. pCMVRev consists of a cytomegalovirus promoter governing expression of a pIIIenv3-1-derived construct (31) from which 583 nt of *env* coding sequences were deleted. Transduced SupT1 cells were electroporated with either pIIIExTat, pCMVRev, or both; 48 h later, total RNA was isolated and examined by Northern blotting analysis using the Neo^r region probe (Fig. 3C).

Introduction of pCMVRev had little effect on vector transcription, while introduction of pIIIExTat resulted in a modest increase in the levels of both the 3.2- and 5.4-kb transcripts. Simultaneous introduction of both *tat* and *rev* expression plasmids into transduced SupT1 cells resulted in a marked increase in the level of unspliced 5.4-kb HIV promoter transcripts, demonstrating that vector gene expression is responsive to HIV-1 Tat and Rev.

This report demonstrates that introduction of an HIV-based retroviral vector into stable HIV-based packaging cell lines yields packaged vectors that transduce a susceptible T-cell line. While the vector titers were extremely low, it is conceivable

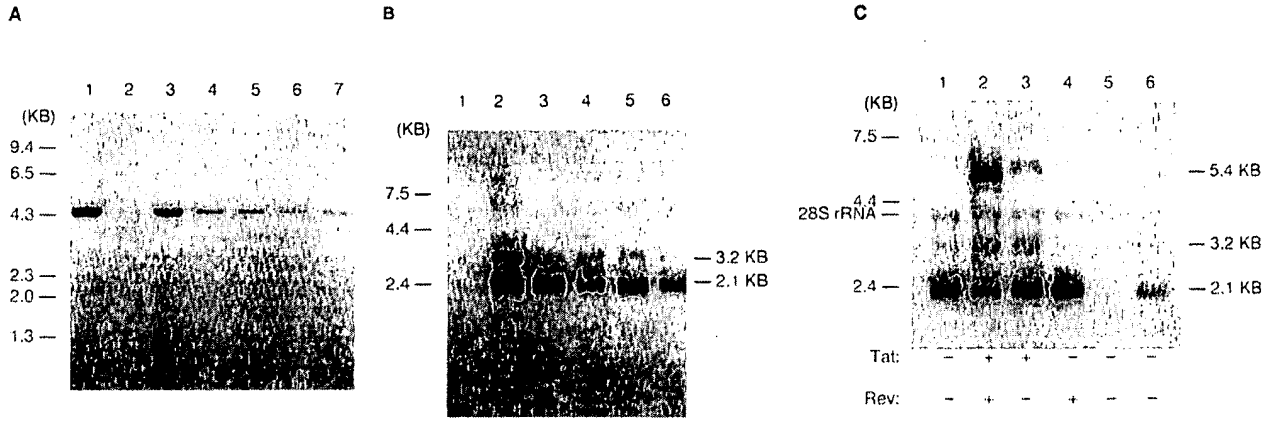


FIG. 3. Characterization of transduced SupT1 cells. (A) Southern blotting analysis of SupT1 cells transduced by supernatant from pooled pHXMoNe-transfected packaging cells. *Sca*I-digested SupT1 genomic DNA was hybridized with a 1-kb Neo^r probe (Fig. 1). Lane 1 contains *Sca*I-digested pHXMoNe vector DNA mixed with genomic DNA from untransduced SupT1 cells. Lane 2 contains genomic DNA from untransduced SupT1 cells. Lanes 3 to 7 contain genomic DNA from transduced SupT1 cells obtained from independent transduction experiments. Molecular sizes are indicated. (B) Northern blotting analysis of SupT1 cells transduced by supernatant from pooled pHXMoNe-transfected packaging cells. RNA isolated from transduced SupT1 cells was hybridized with the Neo^r probe. Lane 1 contains RNA isolated from untransduced SupT1 cells. Lanes 2 to 6 contain SupT1 RNA obtained in independent transduction experiments. Molecular sizes are indicated, as are the sizes of transcripts recognized by the Neo^r probe. (C) Modulation of vector gene expression in transduced SupT1 cells by HIV-1 regulatory gene products. Transduced SupT1 cells were electroporated with the HIV-1 *tat* expression vector pIIIExTat or the *rev* expression vector pCMVRev; 48 h postelectroporation, RNA was isolated and subjected to Northern blotting analysis using the Neo^r probe. Electroporation of *tat* or *rev* expression vectors is indicated. Lanes 1 to 4 contain RNA obtained in separate transduction experiments. Lane 5 contains RNA from untransduced SupT1 cells. Lane 6 contains RNA from a pHXMoNe-transfected packaging cell clone.

that because of the low cloning efficiency of SupT1 cells and the toxic environment created by many untransduced dying cells, many transduced cells failed to grow, resulting in an underestimation of the actual titer.

However, it is likely that other factors, in particular the lack of viral precursor protein processing in packaging cell lysates, contributed directly to the low vector titers. An accumulation of unprocessed precursor proteins in constitutive HIV particle-producing cells has been observed previously, leading the authors to suggest that cells expressing active HIV protease may be placed at a selective disadvantage (14, 17). While virion formation can occur in the absence of precursor protein processing, the released virions are not infectious (16), and they exhibit significantly reduced reverse transcriptase activity (19). This observation is consistent with our finding that supernatant reverse transcriptase activity in both B4.7 and D3.2 cells is markedly reduced and supports the contention that defective protein processing may contribute substantially to the low transducing particle titers obtained.

It is also possible that defects in vector design contributed to the low transducing particle titers. However, a comprehensive analysis of HIV vector packaging requirements showed that vectors containing the ψ site and the RRE, both of which were present in pHXMoNe, are efficiently packaged by wild type HIV-1 (25). Additionally, in the presence of an infectious HIV-1 molecular clone, pHXMoNe can be packaged to titers as high as 10^4 transducing particles per ml (22). These observations suggest that the low vector titers observed in this study are mainly due to defects in the packaging cell line rather than in pHXMoNe.

Distinct pathways can be envisioned by which HIV vectors can provide antiviral defenses. The immune response observed upon administration of HIV-like particles (10–12, 14, 27) may be augmented through the use of HIV vectors expressing immunogens or immunomodulators. Alternatively, HIV-based vectors carrying antiviral agents can enable HIV-susceptible cell populations to mount a tightly regulated, HIV-dependent antiviral response. However, the practicality of any retroviral vector systems depends on the availability of high-titer vector preparations free of replication-competent virus. While methods using transient cotransfection of vector and packaging functions (3, 23, 30) or using wild-type virus to package vector (25) have yielded higher vector titers, vectors prepared by these techniques may be unsuitable for human use protocols. The ability to select and amplify individual stable packaging cells permits the rigorous characterization of homogeneous vector-producing cells. Therefore, although the vector titers that we have reported are low, these data represent a critical step forward in production of a practical HIV-based retroviral vector system ultimately designed for human use.

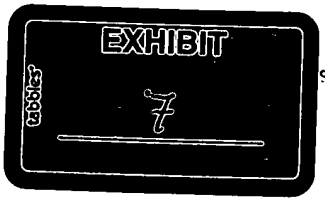
pIIIenv3-1 was obtained from the NIH AIDS Research and Reference Reagent Program (J. Sodroski, donor). We thank C. Rosen and C. Roberts for the generous gifts of pIIIExTat and AIDS patient antisera, respectively. We thank J. Kim, D. Mayers, N. Michael, and M. Vahey for useful discussions and constructive comments on the manuscript.

REFERENCES

1. **Aldovini, A., and R. A. Young.** 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. J. Virol. **64**:1920–1926.
2. **Buchschacher, G. L.** 1993. Molecular targets of gene transfer therapy for HIV infection. JAMA **269**:2880–2886.
3. **Buchschacher, G. L., and A. T. Panganiban.** 1992. Human immunodeficiency virus vectors for inducible expression of foreign genes. J. Virol. **66**:2731–2739.
4. **Clavel, F., and J. M. Orenstein.** 1990. A mutant of human immunodeficiency virus with reduced RNA packaging and abnormal particle morphology. J. Virol. **64**:5230–5234.

5. **Cullen, B. R.** 1992. Mechanism of action of regulatory proteins encoded by complex retroviruses. Microbiol. Rev. **56**:375–394.
6. **Dayton, A. I., E. F. Terwilliger, J. Potz, M. Kowalski, J. G. Sodroski, and W. A. Haseltine.** 1988. Cis-acting sequences responsive to the rev gene product of the human immunodeficiency virus. J. Acquired Immune Defic. Syndr. **1**:441–452.
7. **Eglits, M. A., and W. F. Anderson.** 1988. Retroviral vectors for introduction of genes into mammalian cells. BioTechniques **6**:608–614.
8. **Fisher, A. G., E. Collati, L. Ratner, R. C. Gallo, and F. Wong-Staal.** 1985. A molecular clone of HTLV-III with biological activity. Nature (London) **316**:262–265.
9. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
10. **Griffiths, J. C., S. J. Harris, G. T. Layton, E. L. Berrie, T. J. French, N. R. Burns, S. E. Adams, and A. J. Kingsman.** 1993. Hybrid human immunodeficiency virus Gag particles as an antigen carrier system: induction of cytotoxic T-cell and humoral responses by a Gag:V3 fusion. J. Virol. **67**:3191–3198.
11. **Haffar, O. K., P. A. Moran, M. D. Smithgall, M. L. Diegel, P. Sridhar, J. A. Ledbetter, J. M. Zarling, and S.-L. Hu.** 1992. Inhibition of virus production in peripheral blood mononuclear cells from human immunodeficiency virus (HIV) type 1-seropositive donors by treatment with recombinant HIV-like particles. J. Virol. **66**:4279–4287.
12. **Haffar, O. K., M. D. Smithgall, P. A. Moran, B. M. Travis, J. M. Zarling, and S.-L. Hu.** 1991. HIV-specific humoral and cellular immunity in rabbits vaccinated with recombinant human immunodeficiency virus-like *gag-env* particles. Virology **183**:487–495.
13. **Harrison, G. P., and A. M. L. Lever.** 1992. The human immunodeficiency virus type 1 packaging signal and major splice donor region have a conserved stable secondary structure. J. Virol. **66**:4144–4153.
14. **Haynes, J. R., S. X. Cao, B. Rovinski, C. Sia, O. James, G. A. Dekaban, and M. H. Klein.** 1991. Production of immunogenic HIV-1 viruslike particles in stably engineered monkey cell lines. AIDS Res. Hum. Retroviruses **7**:17–27.
15. **Ho, D. D., R. T. Schooley, T. R. Rota, J. C. Kaplan, T. Flynn, S. Z. Salahuddin, M. A. Gonda, and M. S. Hirsch.** 1984. HTLV-III in the semen and blood of a healthy homosexual man. Science **226**:451–453.
16. **Kohl, N. E., E. A. Emini, W. A. Schlief, L. J. Davis, J. C. Heimbach, R. A. F. Dixon, E. M. Scolnick, and I. S. Sigal.** 1988. Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. USA **85**:4686–4690.
17. **Krausslich, H.-G., C. Ochsenbauer, A.-M. Traenckner, K. Mergener, M. Facke, H. R. Gelderbloom, and V. Bosch.** 1993. Analysis of protein expression and virus-like particle formation in mammalian cell lines stably expressing HIV-1 *gag* and *env* gene products with or without active HIV protease. Virology **192**:605–617.
18. **Lever, A. M. L., H. Gottlinger, W. Haseltine, and J. Sodroski.** 1989. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. J. Virol. **63**:4085–4087.
19. **Mergener, K., M. Facke, R. Welker, V. Brinkmann, H. R. Gelderbloom, and H.-G. Krausslich.** 1992. Analysis of HIV particle formation using transient expression of subviral constructs in mammalian cells. Virology **186**:25–39.
20. **Michael, N. L., M. Vahey, D. S. Burke, and R. R. Redfield.** 1992. Viral DNA and mRNA expression correlate with the stage of human immunodeficiency virus (HIV) type 1 infection in humans: evidence for viral replication in all stages of HIV disease. J. Virol. **66**:310–316.
21. **Miller, A. D.** 1990. Retrovirus packaging cells. Hum. Gene Ther. **1**:5–14.
22. **Mosca, J. D.** Unpublished data.
23. **Poznansky, M., A. Lever, L. Bergeron, W. Haseltine, and J. Sodroski.** 1991. Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. J. Virol. **65**:532–536.
24. **Ratner, L., A. Fisher, L. L. Jagodzinski, H. Mitsuya, R.-S. Liou, R. C. Gallo, and F. Wong-Staal.** 1987. Complete nucleotide sequences of functional clones of the AIDS virus. AIDS Res. Hum. Retroviruses **3**:57–69.
25. **Richardson, J. H., L. A. Child, and A. M. L. Lever.** 1993. Packaging of human immunodeficiency virus type 1 RNA requires *cis*-acting sequences outside the 5′ leader region. J. Virol. **67**:3997–4005.
26. **Rosen, C. A., E. Terwilliger, A. Dayton, J. G. Sodroski, and W. A. Haseltine.** 1988. Intragenic cis-acting art gene-responsive sequences of the human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **85**:2071–2075.
27. **Rovinski, B., J. R. Haynes, S. X. Cao, O. James, C. Sia, S. Zolla-Pazner, T. J. Mathews, and M. H. Klein.** 1992. Expression and characterization of genetically modified human immunodeficiency virus-like particles containing modified envelope glycoproteins: implications for development of a cross-protective AIDS vaccine. J. Virol. **66**:4003–4012.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Schwartz, S., B. K. Felber, D. M. Benko, E.-M. Fenyo, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J. Virol. **64**:2519–2529.
30. **Shimada, T., H. Fujii, H. Mitsuya, and A. W. Nienhuis.** 1991. Targeted and highly efficient gene transfer into CD4+ cells by a recombinant human immunodeficiency virus retroviral vector. J. Clin. Invest. **88**:1043–1047.
31. **Sodroski, J., W. C. Goh, C. Rosen, K. Campbell, and W. A. Haseltine.** 1986. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. Nature (London) **322**:470–474.
32. **Sodroski, J., R. Patarca, C. A. Rosen, F. Wong-Staal, and W. A. Haseltine.** 1985. Location of the trans activating region on the genome of human T-cell lymphotropic virus type III. Science **229**:74–77.
33. **Van Beveren, C., J. G. Goddard, A. Berns, and I. M. Verma.** 1980. Structure of Moloney murine leukemia viral DNA: nucleotide sequence of the 5′ long terminal repeat and adjacent cellular sequences. Proc. Natl. Acad. Sci. USA **77**:3307–3311.

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11382–11388, October 1996
Colloquium Paper



This paper was presented at a colloquium entitled "Genetic Engineering of Viruses and of Virus Vectors," organized by Bernard Roizman and Peter Palese (Co-chairs), held June 9–11, 1996, at the National Academy of Sciences in Irvine, CA.

Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector

LUIGI NALDINI*, ULRIKE BLÖMER, FRED H. GAGE, DIDIER TRONO†, AND INDER M. VERMA

The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, CA 92186-5800

ABSTRACT **We describe the construction of a safe, replication-defective and efficient lentiviral vector suitable for *in vivo* gene delivery. The reverse transcription of the vector was found to be a rate-limiting step; therefore, promoting the reaction inside the vector particles before delivery significantly enhanced the efficiency of gene transfer. After injection into the brain of adult rats, sustained long-term expression of the transgene was obtained in the absence of detectable pathology. A high proportion of the neurons in the areas surrounding the injection sites of the vector expressed the transduced β-galactosidase gene. This pattern was invariant in animals sacrificed several months after a single administration of the vector. Transduction occurs by integration of the vector genome, as it was abolished by a single amino acid substitution in the catalytic site of the integrase protein incorporated in the vector. Development of clinically acceptable derivatives of the lentiviral vector may thus enable the sustained delivery of significant amounts of a therapeutic gene product in a wide variety of somatic tissues.**

Gene therapy is a promising new form of medicine because of its potential to reverse the genetic causes of several innate and acquired diseases (1, 2). The currently available methods of gene delivery suffer from several major limitations that curtail the realization of these high expectations. Nonviral methods are inefficient and only attain a transient expression of the transgene, while no viral vector yet offers a satisfactory combination of efficacy of gene transfer, sustained transgene expression, and biosafety (3, 4). Adenoviral vectors allow highly efficient delivery of the transgene in most tissues *in vivo*, but its expression is transient. This is mostly due to the immune response against the transduced cells, which also express a low level of viral proteins (5–9). Vectors derived from oncoretroviruses, such as the Moloney leukemia virus (MLV), integrate the transgene in the genome of the target cells without transferring any viral gene, two properties considered crucial for the sustained expression of the transgene (10). These prototypic retroviral vectors, however, are severely restricted in their potential targets, as they only transduce cells that divide shortly after infection (11). Consequently, they are most often employed in demanding *ex vivo* protocols of gene transfer (12–16). Furthermore, transcriptional shutoff of the transgene after reimplantation *in vivo* of the transduced cells is frequently observed (17, 18).

We have previously described a human lentivirus (HIV)-based vector that can transduce nondividing cells. As the particles are pseudotyped with the envelope of the vesicular stomatitis virus (VSV), the vector can serve to introduce genes into a broad range of tissues and can be used *in vivo* (19). Furthermore, we demonstrate that *in vivo* gene transfer is dependent on a functional integrase protein and that transgene expression is sustained for several months without detectable pathology. These characteristics suggest that lentiviral vectors could play a major role in the arena of gene therapy.

MATERIALS AND METHODS

Plasmid Construction. The construction of the HIV-derived plasmids pCMVΔR9, pHR'-CMVLacZ, and pHR'-CMVLucif has been described (19). Plasmid pCMVΔR8.2 was derived from pCMVΔR9 by substituting, for a 2.7-kbp *Sal*I–*Bam*HI fragment, a 0.5-kbp *Sal*I–*Not*I fragment (obtained by a PCR that added to the proviral HIV-1 NL4-3 DNA sequence, at the end of the *vpu* gene, a stop codon in the *env* reading frame followed by a *Not*I site) and a 0.85-kbp *Not*I–*Bam*HI fragment from pHR', containing a *Not*I linker introduced at the *Bgl*II site 7620 in the HIV-1 HXB2D sequence, the Rev responsive element and the splice acceptor site for the second exon of the *tat* and *rev* genes. The construction deletes nucleotides 6308–7611 of the HIV-1 NL4-3 genome, encompassing a large portion of the *env* coding sequence, from the packaging plasmid. Its remaining HIV-1 sequences are derived from plasmid pR7 (20) [nucleotides 708–1506 and 7620–9416 in the HXB2D sequence (21)] and NL4.3 (nucleotides 1507–6307). Plasmid pMD.G drives the expression of the VSV.G reading frame from the human cytomegalovirus immediate-early promoter (hCMV) and contains β-globin sequences upstream (exons 2 and 3, intervening sequence 2) and downstream (polyadenylylation site) of it. All plasmids contain the simian virus 40 origin of replication in the backbone.

Production and Assays of Vectors. Human kidney 293T cells (1.5×10^6) were plated on 10-cm plates and transfected the following day with 15 μg of pCMVΔR8.2, 20 μg of either pHR' plasmid, and 5 μg of pMD.G by calcium phosphate DNA precipitation (22). Conditioned medium was harvested 62 hr after transfection, cleared of debris by low-speed centrifugation, filtered through 0.45-μm filters, and assayed for p24 Gag antigen by ELISA (DuPont). For transduction of rat 208F fibroblasts, cells were infected overnight with serial dilutions of vector stock in culture medium supplemented with 8 μg of polybrene per ml. After medium replacement, the cells were further incubated for 36 hr, and expression of β-galactosidase (β-gal) was scored by 5-bromo-4-chloro-3-indolyl β-D-galac-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MLV, Moloney leukemia virus; VSV, vesicular stomatitis virus; hCMV, human cytomegalovirus immediate-early promoter; β-gal, β-galactosidase.

*Present address: Somatix Therapy Corporation, 850 Marina Village Parkway, Alameda, CA 94501.
†To whom reprint requests should be addressed. e-mail: didier_trono@qm.salk.edu

toside (X-Gal) staining. Titers were calculated by counting the number of foci of blue cells per well and dividing it by the dilution factor. Expression of luciferase was assayed by washing the cultures twice with TBS (50 mM Tris·HCl, pH 7.8/130 mM NaCl/10 mM KCl/5 mM $MgCl_2$), extracting them with 0.5% Nonidet P-40 in TBS containing 1 mM dithiotreithol, and measuring luminescence in a luminometer. Concentrated vector stocks were prepared by ultracentrifugation of conditioned medium at 50,000 × *g* for 90 min, resuspension of the pellets in a small volume (half percent of the starting volume of medium) of TBS containing 10 mM $MgCl_2$, pooling, and incubation with or without 0.1 mM of each of the four deoxynucleotides (dNTPs), 3 mM spermine, and 0.3 mM spermidine for 2 hr at 37°C. After dilution in TBS, the vector particles were concentrated by a second ultracentrifugation, and the final pellet was resuspended in a very small volume (half thousandth of the starting volume of medium) of sterile saline containing 4 μg of polybrene per ml. Resuspension of the second pellet required prolonged incubation and pipetting. Stocks were stored frozen at −80°C and titered before and after freezing. MLV-based β-gal vector was similarly produced by the transient transfection into 293T cells of a plasmid driving the MLV *gag* and *pol* genes from the hCMV promoter (the vector pLNL-CMVLacZ, which carries the same β-gal expression cassette as the HIV-based vector) and the pMD.G plasmid. Before injection, all batches of vector were tested for the absence of replication-competent virus by infecting HeLa cells at high multiplicity of infection (HeLa P4 cells, which express CD4 and contain an integrated LacZ gene driven by the HIV long terminal repeat), obtained from the American Type Culture Collection stock (23), and HeLa cells previously transduced with a lentiviral vector carrying a different reporter gene and selected for its expression. The transduced cells were passaged two to three times, and the conditioned medium was tested for transfer of the markers to virgin 208F fibroblasts or for β-gal-inducing activity on P4 cells.

***In Vivo* Delivery of Vectors and Immunostaining of Sections from the Injected Brains.** All animal procedures were performed according to an institution-approved protocol and under strict biological containment. Adult female Fischer 344 rats were anesthetized (ketamine, 44 mg/kg; acepromazine, 0.75 mg/kg; and xylazine, 4 mg/kg, in 0.9% NaCl i.p.) and positioned in a stereotactic head frame. After midline incision of the skin, holes were drilled in the appropriate locations in the skull with a dental bur, and a 5-μl Hamilton syringe with needle was used to slowly inject 2 μl of vector suspension in sterile saline into the striatum (AP +0.2, ML ± 3.5, DV −4.5) and hippocampus (AP −3.5, ML 3.0, DV −4.0) bilaterally. Holes were then filled with bone wax, the incision was sutured, and the animals were returned to their cages. For assessing transduction, the rats were deeply anesthetized and perfused with 4% cold paraformaldehyde and 0.2% glutaraldehyde intracardially. The brains were removed, post-fixed at 24 hr, saturated in 30% sucrose, and sectioned on a freezing microtome (50-μm serial sections). For light microscopy, sections were incubated with rabbit anti-β-gal antibodies (1:1000; Cortex Pharmaceuticals, Irvine, CA) and stained using avidin–biotin–peroxidase (Vectastain ABC Elite; Vector Laboratories) and diaminobenzidine. For immunofluorescence labeling, mouse monoclonal anti-NeuN and guinea pig anti-GFAP antibodies, secondary antibodies coupled to the fluorescent markers CY5, dichlorotriazinyl amino fluorescein, and Texas Red were also used, and the mounted sections were analyzed by confocal scanning laser microscopy (Bio-Rad model MRTC600). Fluorescent signals were collected, digitally color enhanced, and superimposed.

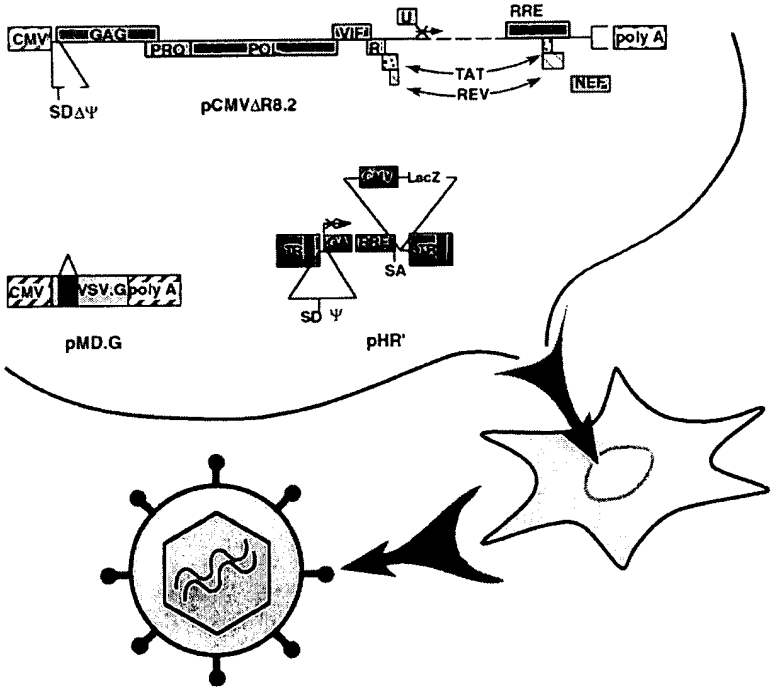


FIG. 1. Schematic of the generation of lentiviral vector. The relevant portions of the three plasmids cotransfected into 293T cells are depicted at the top; their contribution to the vector particles harvested in the transfectant conditioned medium are depicted at the bottom. pCMVΔR8.2, the packaging construct, provides all vector proteins but the envelope. The viral genes are expressed from the hCMV promoter and the insulin polyadenylylation site. Proviral 5′ leader and Ψ sequences have been deleted together with a large portion of the *env* gene. A translation stop codon was inserted upstream of the remaining *env* segment. The major 5′ splice donor site (SD) has been conserved. pHR′, the transducing vector, provides the vector genome. The viral long terminal repeats (LTRs) and the Ψ sequence are indicated. The *gag* gene is truncated after 350 bp and is out of frame (×), and it follows the Rev responsive element (RRE) and a splice acceptor site (SA). The position of a hCMV-driven expression cassette for the β-gal cDNA (LacZ) is shown. pMD.G encodes the heterologous VSV envelope that pseudotypes the vector. The hCMV promoter drives the VSV.G reading frame, which has β-globin sequences upstream (exons 2 and 3, intervening sequence 2) and downstream [poly(A) site].

RESULTS

Generation of High-Titer Lentiviral Vector with Improved Biosafety. The lentiviral vector is produced by the transient transfection of three plasmids into human kidney 293T cells (Fig. 1). Two complementary constructs were derived from the HIV-1 proviral DNA. The packaging construct expresses all HIV *trans*-acting proteins but the envelope from heterologous transcription signals. The transducing vector, pHR', retains all HIV *cis*-acting sequences required for its transfer to the target cell, now framing an expression cassette for the transgene. The *Escherichia coli* β-gal gene and the firefly luciferase gene driven by the hCMV promoter were used as reporter genes in this study. A novel packaging plasmid, pCMVΔR8.2, was constructed to improve the biosafety of the vector. It was derived from the previously described plasmid pCMVΔR9 by deletion of 1.4 kbp from the *env* gene sequence, downstream of the (functional) *vpu* gene, and substitution with an inframe stop codon. The deletion of *env* sequences did not affect the yield or the transduction efficiency of the vector particles. The third plasmid, pMD.G, encodes the heterologous VSV envelope and is used for pseudotyping the particles generated by the other two constructs.

High-titer stocks were obtained by pelleting the vectors from the transfectants conditioned medium by two rounds of ultracentrifugation at 50,000 × *g* for 90 min, as described for VSV.G-pseudotyped MLV vectors (24). Vector yield averaged 50% for each centrifugation step, both measured as p24 Gag equivalent and as transducing units in 208F rat fibroblasts. The overall yield of the protocol was ≈25%, with an increase in transducing titer of three orders of magnitude. As the transfectant conditioned medium contained, on average, 4×10^5 transducing units/ml, titers of $2–4 \times 10^8$ transducing units/ml were routinely achieved. When normalized to the content of p24 Gag antigen, the transducing activity of the vector was not affected by the centrifugation steps, averaging 4500 transducing units per ng of p24 in 208F fibroblasts. The absence of replication-competent virus from all vector stocks was proven by the lack of spreading or mobilization of reporter genes from transduced cells (see *Materials and Methods*).

Transduction Is Enhanced by Promoting Intraparticle Reverse Transcription. We previously showed that the HIV-based vector is less efficient in cells arrested in G_0 than in cells growing or arrested in G_1/S or G_2. This became more marked the longer the culture had been in G_0 and correlated with a progressive block in reverse transcription of the vector genome (19). We decided to test whether promotion of the reaction inside the vector particle, as described by Zhang *et al.* (25) for the HIV virus, would enhance its transducing activity. Upon incubation with a mixture of the four dNTPs and the polyamine spermine and spermidine for 2 hr at 37°C, the transduction of a luciferase reporter gene increased 2-fold in growing 208F fibroblasts and up to 5-fold in G_0-arrested cells, becoming independent of the length of growth-arrest of the culture (Fig. 2). We then tested whether a similar effect was observed in the transduction of terminally differentiated cells *in vivo*.

High Efficiency of Transduction by the Lentiviral Vector of Rat Brain Neurons *in Vivo*. Stocks of lentiviral β-Gal vector, matched for the content of p24 Gag antigen, were incubated with or without dNTPs and polyamines for 2 hr at 37°C before injection. β-Gal MLV-based vector, pseudotyped with the VSV envelope, was also treated with the dNTPs and matched for transducing activity on 208F cells. Vector suspension (2 μl) was injected into the corpus striatum and hippocampus of both sides of the brain of anesthetized adult female Fisher rats. Groups of three animals each were sacrificed at increasingly longer time intervals (2 weeks, 6 weeks, 3 months) from a single vector administration. Transduction rate was assessed for each injected brain by serial cryostatic sectioning, and immunostaining of each sixth section for β-gal. The relative proportion of transduced cell types was estimated by immunofluorescence costaining of representative sections with antibodies directed against β-gal and cell type-specific markers and confocal microscope analysis.

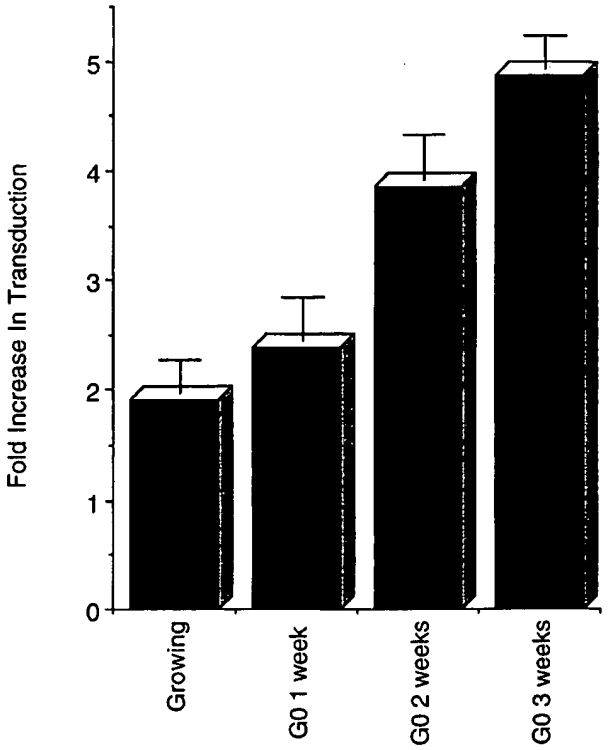


FIG. 2. Transduction is enhanced by promoting reverse transcription inside the lentiviral vector before infection. Rat 208F cells were plated at low density and either infected the following day (Growing) or grown to confluence, switched to medium containing 5% calf serum and 2 μM dexamethasone (11), and further incubated for the indicated number of weeks before infection (G0 *x* weeks, where *x* = number of weeks) with lentiviral luciferase vector preincubated with or without dNTPs and polyamines. Transduction was scored by comparing luminescence in cell extracts 48 hr after infection with both vectors. Plotted is the mean ± SEM increase in transduction by the treated vector over the level induced by the untreated vector, calculated from infections performed at three different multiplicities of infection over a hundred-fold range in a typical experiment. In the cells arrested in G_0, the transduction by the untreated vector was 28% (G0 1 week), 18% (G0 2 weeks), and 13% (G0 3 weeks) of that scored in growing cells.

FIG. 3 (*on opposite page*). Immunohistochemical staining for β-gal of sections from the corpus striatum (*Left*) or the hippocampus (*Right*) of rat brains injected with a single dose of β-gal vector 2 weeks or 3 months before. The viral parent of the injected vector is indicated on the left, as also is the pretreatment of vector with dNTPs and polyamines. The HIV integrase mutant carries a single amino acid replacement in the catalytic site (D64V), which severely reduces the activity of the enzyme incorporated in the vector. Note the lower magnification in the three bottom rows. The lentiviral vector achieves an efficient gene transfer into cells of typical neuronal morphology; the transduction is significantly enhanced by the pretreatment with dNTPs and is abolished by the integrase mutation. No change in the pattern and density of β-gal-expressing cells is observed even after 3 months. In contrast, the MLV-derived vector shows a poor gene transfer, exclusively in cells of glial morphology, and no expression is detected after 3 months. The sections shown are representative of those obtained in the proximity of each injected site in all animals in the experimental group. One of every six serial sections from the injected brains was stained, and transduced cells could be detected in up to six stained sections, for the lentiviral vector treated with dNTPs.

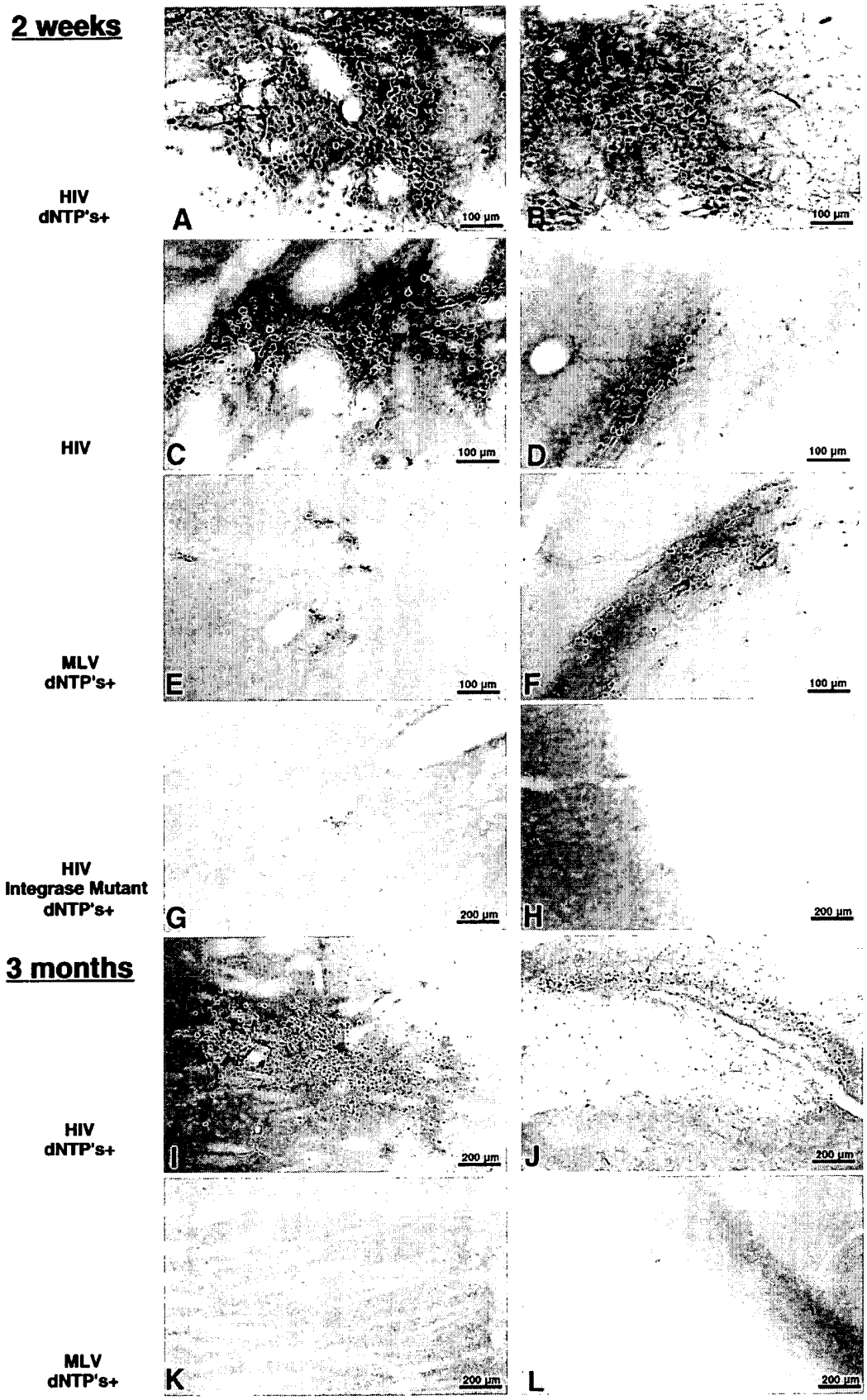


FIG. 3. (*Legend appears on opposite page.*)

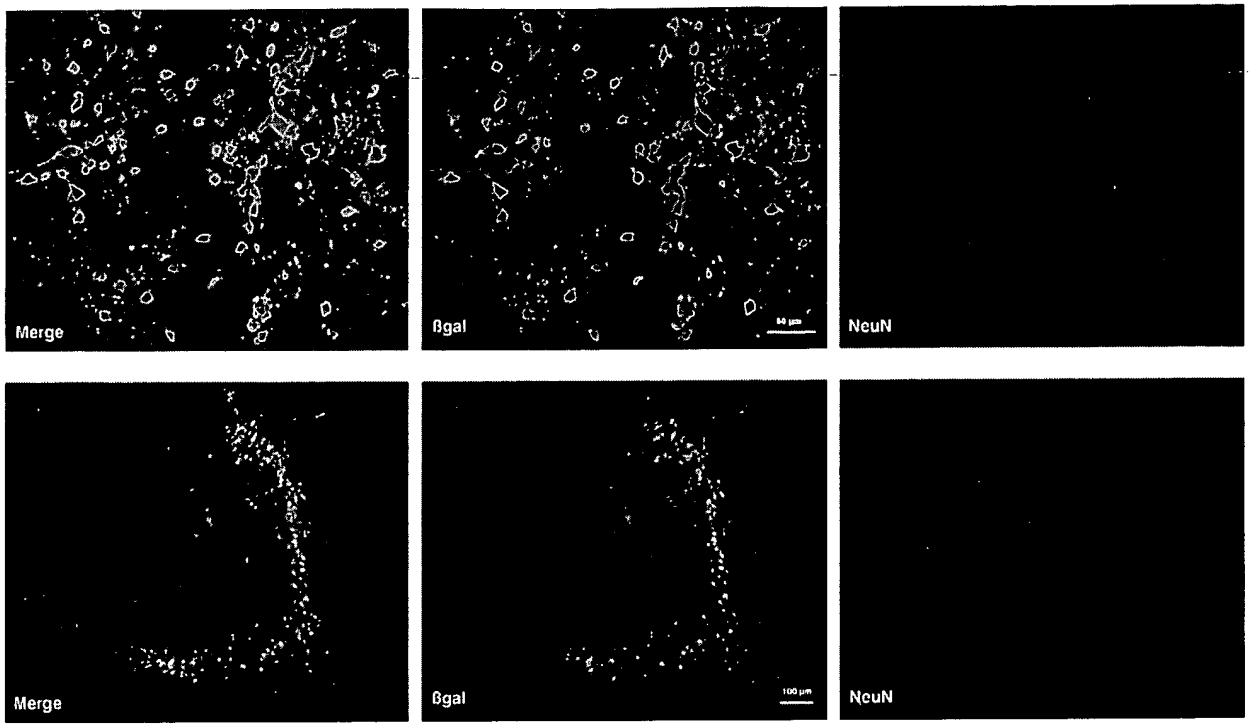


FIG. 4. Immunofluorescence staining for β-gal and the neuronal marker NeuN of sections from the corpus striatum (*Upper*) and the hippocampus (*Lower*) of a rat brain injected 6 weeks earlier with lentiviral β-gal vector pretreated with dNTPs. Shown are confocal microscope images obtained from each individual staining (*Middle* and *Right*) and their overlap (*Left*). A significant fraction of the neurons in the area surrounding the injection site expresses the transgene.

A conspicuous fraction of cells was reproducibly transduced by the lentiviral vector around the injection site, and cells expressing β-gal could be detected up to several millimeters away from it, as shown in Fig. 3 *A*, *B*, *I*, and *J*. The majority of transduced cells showed neuronal morphology and, when costained for immunofluorescence, expressed the neuronal marker protein NeuN (ref. 26; Fig. 4). Treatment of the lentiviral vector with dNTPs before delivery enhanced significantly its transduction rate *in vivo* (Fig. 3, compare *A* and *B* with *C* and *D*). An estimate of the average density of transduced cells indicated at least a 2-fold increase (data not shown). The MLV-based vector displayed a comparatively poor transduction rate (Fig. 3 *E* and *F*). Cells transduced by the MLV vector were smaller and different from those predominantly stained in lentiviral-injected brains and looked like oligodendrocytes and astrocytes. As expected, none could be found by immunofluorescence analysis that expressed β-gal together with the neuronal marker NeuN (data not shown).

Long-Term Expression of the Transgene *in Vivo* with No Detectable Pathology. The pattern and the estimated density of cells expressing β-gal in the injected areas did not change appreciably with the time elapsed since administration of the lentiviral vector. This remained true for 3 months, the longest time examined (Fig. 3 *I* and *J*). Occasionally, some detectable tissue damage in the injection site was observed at the earlier times after injection, possibly related to local bleeding, but the majority of the examined tissues were remarkably unaffected by the delivery and expression of the transgene, as also seen by hematoxylin and eosin staining (data not shown). Furthermore, the distribution of glial cells was not altered in the injected areas, as documented by immunofluorescent staining for the expression of the astrocytic marker glial fibrillary acidic protein (data not shown). In brains injected with the MLV-based vector and examined at late times after administration, almost no cells were detected that still expressed the transgene (Fig. 3 *K* and *L*).

Transduction *in Vivo* Occurs by Integration of the Transgene. To verify whether transduction *in vivo* occurs by integration of the transgene, we used a β-gal vector incorporating a mutant form of integrase carrying a single amino acid substitution (D64V) in the catalytic site. The generation and biological properties of this mutation, both in the context of the HIV virus and the lentiviral vector, have been described (27),(19). While the mutation severely decreases the activity of the enzyme *in vitro* and *in vivo*, it has no detectable effect on the preceding steps of the infection pathway, including particle budding, entry into the target cell, reverse transcription, and nuclear import. It did, though, reduce transduction by the β-gal vector *in vitro* to a residual activity <2% of that of the wild-type. For testing its effect *in vivo*, a concentrated stock of vector incorporating the mutant enzyme was prepared as above, matched to the wild-type for content of p24 Gag antigen, and injected into the brain of three rats. In all injected sites, examined either after 2 or 6 weeks, from none to a couple of rare cells could be detected expressing β-gal, providing genetic evidence that expression of the transgene *in vivo* depends on its integration (Fig. 3 *G* and *H*).

DISCUSSION

The design of a viral vector system relies upon the segregation in the viral genome of *cis*-acting sequences involved in its transfer to target cells from *trans*-acting sequences encoding the viral proteins. The prototype vector particle is assembled by viral proteins expressed from constructs stripped of all *cis*-acting sequences. These sequences are instead used to frame the expression cassette for the transgene driven by an heterologous promoter. As the particle will transfer only the latter construct, the infection process is limited to a single round without spreading. The safety and efficiency of an actual vector system depends on the extent to which this ideal, complete segregation of *cis*- and *trans*-acting functions is obtained.

In the case of HIV, several sequences have been implicated by deletion studies in the encapsidation and dimerization of viral RNA. In contrast to MLV, where the Ψ sequence, located in the 5′-untranslated leader downstream of the major splice donor site, strongly contributes to RNA packaging, the role of the corresponding region in the HIV genome seems more to discriminate genomic from spliced transcripts than to promote efficient encapsidation (28–30). Additional, and possibly more important, sequences have been identified in the transcribed long terminal repeats and 5′ leader sequences upstream of the major splice donor site (31–35). A major caveat, however, is that reconstitution of the packaging function has not yet been reported by juxtaposition of the identified sequence(s) to heterologous RNA (36). Thus, the packaging signal of HIV is either highly sequence-specific or, more likely, multipartite and distributed over a rather large stretch of its 5′ sequence (37).

Several features of the HIV-derived packaging plasmid described here prevent its transfer to the target cells. The combined modifications of the 5′ end delete or disrupt all structural motifs to date implicated in RNA encapsidation and dimerization, with the possible exception of the 5′ portion of the *gag* sequence (30, 38, 39). Recently, McBride and Panganiban (35) reported the encapsidation efficiency of HIV-1 transcripts carrying deletions of the 5′ leader sequence; relative to the wild-type counterpart, it was reduced to <0.1 for transcripts derived from a construct comparable to pCMVΔR8.2 and to <0.02 in the presence of competing wild-type RNA (35). Furthermore, the deletion of both long terminal repeats and of the primer binding site from the packaging plasmid would prevent reverse transcription and integration of any encapsidated transcript not recombined with the vector RNA. The transducing vector, on the other hand, is endowed with a full complement of the *cis*-acting sequences not identified until now, which allows its proficient transfer to the target cell.

It is well recognized that the retroviral infection is an inefficient process. Once the content of virions is delivered inside the target cell, uncoating, reverse transcription, interaction with cytoplasmic chaperones and the nuclear import machinery, and maturation to an integration-competent complex take place. These events, the mechanism of which is still poorly understood, can result in degradation and arrest at a stable intermediate, as well as integration of the viral genome (40). Partial reverse transcripts have been detected in HIV and MLV virions (41–43). Recently, it was shown that viral DNA synthesis can be promoted inside intact HIV-1 particles by exposure to dNTPs and magnesium chloride and that the efficiency of the reaction can be increased by the addition of the polyamine spermine and spermidine (25, 44, 45). The resulting HIV-1 virions exhibit an increased infectivity in primary T lymphocytes infected before activation, a setting in which reverse transcription was previously demonstrated to be a rate-limiting step (46–48). The stimulation of reverse transcription inside virions was also shown to increase the transduction efficiency of MLV-based retroviral vectors in dividing targets (49). Here, we find that it significantly augments the efficacy of gene transfer mediated by the lentiviral vector. This effect was most pronounced in nondividing cells and could also be observed *in vivo*. Performing such *in vitro* reverse transcription reactions before injecting the vector may be critical for some nonproliferating targets that maintain low cytoplasmic pools of dNTPs (50, 51).

The crucial advantage of the lentiviral vector is its integration in the genome of nondividing cells. This was proven here by the dependence of the transduction on the incorporation of a functional integrase in the vector. At least in the case of the brain, the only tissue studied so far, this provides for long-term sustained expression of the transgene. No decrease in the extent of β-gal immunoreactivity was observed even 3 months after a single vector administration. Given the recent report of predominant transgene-directed immune responses in animals transduced with adenoviral vectors (52), it remains to be determined whether the brain represents an immune haven, as long suspected, or whether the adenoviral proteins expressed by the transduced cells played a critical adjuvant role.

Retroviruses are thought to select, through poorly understood mechanisms, active chromatin sites for the integration of their genome (40). This may explain why gene delivery methods based on MLV-derived vectors often suffer from the transcriptional shutoff of the transgene, as was observed in this study, when the transduced cells return to a nonproliferating status and presumably revise their pattern of chromatin expression. The ability of the lentiviral vector to integrate in nondividing cells may allow for the selection of stably open chromatin sites, thus ensuring against the transcriptional silencing of the transgene.

The high prevalence of neurons observed among the transduced cell types in the brain may be due both to the neurotropism of the envelope of the VSV, a rhabdovirus (53), and to a preferential expression of the hCMV promoter in neurons, as recently observed with transgenic animals (54). It may also reflect preferential long-term expression in nondividing cells, for the reasons discussed above.

A major issue now concerns the biosafety of the lentiviral vector. The novel feature of the packaging plasmid described in this paper precludes the generation of wild-type HIV viruses, even by unlikely rearrangement and recombination events, given the actual absence of most of HIV *env* sequences in all three plasmids. In the previously described plasmid pCMVΔR9, the *env* reading frame was blocked by insertion of a linker containing multiple stop codons. The use of a separate plasmid encoding a heterologous envelope makes it extremely unlikely that a replication-competent recombinant be generated. This would require multiple recombination events between different plasmids and/or endogenous retroviral sequences, including recombination between nonhomologous sequences. Careful scrutiny and improvement of the vector-producing system, including evaluation of the minimal set of viral genes required for efficient packaging of the vector and generation of stable packaging systems better amenable to monitoring, are now required.

We are extremely grateful to R. Pomerantz and colleagues for communicating their results before publication. We thank members of the Verma, Gage, and Trono laboratories for helpful suggestions. L.N. was supported by the American–Italian Cancer Foundation, and U.B. was supported by the Deutsche Forschungsgemeinschaft (DFG). This work is supported by grants from the National Institutes of Health and the American Cancer Society (I.M.V.), U.S. Public Health Service Grant AI 37510 (D.T.); National Institutes of Health Grants AG10435 and 08514 and Hollfelder Foundation (F.H.G.); and the H. N. and Frances Berger Foundation (D.T., I.M.V., and F.H.G.). I.M.V. is an American Cancer Society Professor of Molecular Biology and D.T. is a Pew Scholar.

1. Mulligan, R. C. (1993) *Science* **260**, 926–932.
2. Verma, I. (1994) *Mol. Med.* **1**, 2–3.
3. Crystal, R. G. (1995) *Science* **270**, 404–410.
4. Leiden, J. M. (1995) *New Engl. J. Med.* **333**, 871–873.
5. Yang, Y., Li, Q., Ertl, J. & Wilson, J. M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 4407–4411.
6. Yang, Y., Ertl, J. & Wilson, J. M. (1994) *Immunity* **1**, 433–442.
7. Dai, Y., Schwarz, E. M., Gu, D., Zhang, W.-W., Sarvetnick, N. & Verma, I. M. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 1401–1405.
8. Knowles, R. M., Hohneker, K. W., Zhou, Z., Olsen, J. C., Noah, T. L., Hu, P.-C., Leigh, M., Engelhardt, J. F., Edwards, L. J., Jones, K. R., Grossman, M., Wilson, J. M., Johnson, L. G. & Boucher, R. C. (1995) *New Engl. J. Med.* **333**, 823–831.
9. Kremer, E. J. & Perricaudet, M. (1995) *Br. Med. Bull.* **51**, 31–44.
10. Miller, A. D., Miller, D. G., Garcia, J. V. & Lynch, C. M. (1993) *Methods Enzymol.* **217**, 581–599.

11. Miller, D. G., Adam, M. A. & Miller, A. D. (1990) *Mol. Cell. Biol.* **10,** 4239–4242.
12. Dai, Y., Roman, M., Naviaux, R. K. & Verma, I. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10892–10895.
13. Blaese, R. M., Culver, K. W., Miller, A. D., Carter, C. S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., Greenblatt, J. J., Rosenberg, S. A., Klein, H., Berger, M., Mullen, C. A., Ramsey, W. J., Muul, L., Morgan, R. A. & Anderson, W. F. (1995) *Science* **270,** 475–480.
14. Bordignon, C., Notarangelo, L. D., Nobili, N., Ferrari, G., Casorati, G., Panina, P., Mazzolari, E., Maggioni, D., Rossi, C., Servida, P., Ugazio, A. G. & Mavilio, F. (1995) *Science* **270,** 470–475.
15. Kohn, D. B., Weinberg, K. J., Nolta, J. A., Heiss, L. N., Lenarski, C., *et al.* (1995) *Nat. Med.* **1,** 1017–1023.
16. Grossman, M., Radar, D. J., Muller, D. W. M., Kolansky, D. M., Kozarsky, K., Clark, B. J. I., Stein, E. A., Lupien, P. J., Brewer, H. B., Raper, S. E. & Wilson, J. M. (1995) *Nat. Med.* **1,** 1148–1154.
17. Scharfmann, R., Axelrod, J. H. & Verma, I. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4626–4630.
18. Palmer, T. D., Rosman, G. J., Osborne, W. R. A. & Miller, A. D. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1330–1334.
19. Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M. & Trono, D. (1996) *Science* **272,** 263–267.
20. Trono, D., Feinberg, M. B. & Baltimore, D. (1989) *Cell* **59,** 113–120.
21. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Donan, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Gharayeb, J., Chang, N. T., Gaho, R. C. & Wong-Staal, F. (1985) *Nature (London)* **313,** 277–284.
22. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752.
23. Charneau, P., Mirambeau, G., Roux, P., Paulous, S., Buc, H. & Clavel, F. (1994) *J. Mol. Biol.* **241,** 651–662.
24. Burns, J. C., Friedman, T., Driever, W., Burrascano, M. & Yii, J.-K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037.
25. Zhang, H., Dornadula, G. & Pomerantz, R. J. (1996) *J. Virology* **70,** 2809–2824.
26. Mullen, R. J., Buck, C. R. & Smith, A. M. (1992) *Development (Cambridge, U.K.)* **116,** 201–211.
27. Leavitt, A. D., Robles, G., Alesandro, N. & Varmus, H. E. (1996) *J. Virol.* **70,** 721–728.
28. Lever, A., Gottlinger, H., Haseltine, W. & Sodroski, J. (1989) *J. Virol.* **63,** 4085–4087.
29. Aldovini, A. & Young, R. A. (1990) *J. Virol.* **64,** 1920–1926.
30. Luban, J. & Goff, S. P. (1994) *J. Virol.* **68,** 3784–3793.
31. Kim, H.-J., Lee, K. & O'Rear, J. J. (1994) *Virology* **198,** 336–340.
32. Vicenzi, E., Dimitrov, D. S., Engelman, A., Migone, T.-S., Purcell, D. F. J., Leonard, J., Englund, G. & Martin, M. A. (1994) *J. Virol.* **68,** 7879–7890.
33. Geigenmuller, U. & Linial, M. L. (1996) *J. Virol.* **70,** 667–671.
34. Paillart, J.-C., Skripkin, E., Ehresmann, B., Ehresmann, C. & Marquet, R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5572–5577.
35. McBride, M. S. & Panganiban, A. (1996) *J. Virol.* **70,** 2963–2973.
36. Berkowitz, R. D., Hammarskjöld, M.-L., Helga-Maria, C., Rekosh, D. & Goff, S. P. (1995) *Virology* **212,** 718–723.
37. Kaye, J. F., Richardson, J. H. & Lever, A. M. L. (1995) *J. Virol.* **69,** 6588–6592.
38. Buchschacher, G. L. J. & Panganiban, A. T. (1992) *J. Virol.* **66,** 2731–2739.
39. Parolin, C., Dorfman, T., Palú, G., Göttlinger, H. & Sodroski, J. (1994) *J. Virol.* **68,** 3888–3895.
40. Coffin, J. M. (1990) in *Virology*, eds. B. N. Fields & D. M. Knipe (Raven, New York), pp. 1437–1500.
41. Trono, D. (1992) *J. Virol.* **66,** 4893–4900.
42. Lori, R., di Marzo Veronese, F., De Vico, A. L., Lusso, P., Reitz, M. S., Jr., & Gallo, R. C. (1992) *J. Virol.* **66,** 5067–5074.
43. Zhu, J. & Cunningham, J. M. (1993) *J. Virol.* **67,** 2385–2388.
44. Zhang, H., Zhang, Y., Spicer, T. P., Abbott, L. Z., Abbott, M. & Poiesz, B. J. (1993) *AIDS Res. Hum. Retroviruses* **9,** 1287–1296.
45. Zhang, H., Zhang, Y., Spicer, T., Henrard, D. & Poiesz, B. (1995) *J. Virol.* **69,** 3675–3682.
46. Zack, J. A., Haislip, A. M., Krogstad, P. & Chen, I. S. Y. (1992) *J. Virol.* **66,** 1717–1725.
47. Gao, W.-Y., Cara, A., Gallo, R. C. & Lori, F. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8925–8928.
48. Meyerhans, A., Vartanian, J.-P., Hultgren, C., Plikat, U., Karlsson, A., Wang, L., Eriksson, S. & Wain-Hobson, S. (1994) *J. Virol.* **68,** 535–540.
49. Zhang, H., Duan, L.-X., Dornadula, G. & Pomerantz, R. J. (1995) *J. Virol.* **69,** 3929–3932.
50. Cohen, A., Barankiewica, J., Lederman, H. M. & Gelfand, E. W. (1983) *J. Biol. Chem.* **258,** 12334–12340.
51. McCormick, P. J., Danhauser, L. L., Rustum, Y. M. & Bertram, J. S. (1983) *Biochem. Biophys. Res. Comm.* **755,** 36–40.
52. Tripathy, S. K., Black, H. B., Goldwasser, E. & Leiden, J. M. (1996) *Nat. Med.* **2,** 545–550.
53. Wagner, R. R. (1987) in *Rhabdovirus Biology and Infection: An Overview*, ed. Wagner, R. R. (Plenum, New York), pp. 9–74.
54. Baskar, J. F., Smith, P. P., Nilaver, G., Jupp, R. A., Hoffmann, S., Peffer, N. J., Tenney, D. J., Colberg-Poley, A. M., Ghazal, P. & Nelson, J. A. (1996) *J. Virol.* **70,** 3207–3214.